Analyst

Accepted Manuscript

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/analyst

Making a big thing of a small cell – recent advances in single cell analysis

Kerstin Galler [1,2], Katharina Bräutigam [2,3], Christina Große [1,2], Jürgen Popp [1,2,3], Ute Neugebauer [1,2]

[1] Integrated Research and Treatment Center "Center for Sepsis Control and Care", Jena University Hospital, Erlanger Allee 101, 07747 Jena, Germany

[2] Leibniz Institute of Photonic Technology Jena, Albert-Einstein-Straße 9, 07745 Jena, Germany

[3] Institute of Physical Chemistry and Abbe Center of Photonics, Friedrich Schiller University, Helmholtzweg 4, 07743 Jena, Germany

Abstract

Single cell analysis is an emerging field requiring a high level interdisciplinary collaboration to provide detailed insights into the complex organisation, function and heterogeneity of life. This review is addressed to life science researchers as well as researchers developing novel technologies. It covers all aspects of the characterisation of single cells (with a special focus on mammalian cells) from morphology to genetics and different omics-techniques to physiological, mechanical and electrical methods. In recent years, tremendous advances have been achieved in all fields of single cell analysis: 1. improved spatial and temporal resolution of imaging techniques to enable the tracking of single molecule dynamics within single cells; 2. increased throughput to reveal unexpected heterogeneity between different individual cells raising the question what characterizes a cell type and what is just natural biological variation; and 3. emerging multimodal approaches try to bring together information from complementary techniques paving the way for a deeper understanding of the complexity of biological processes. This review also covers the first successful translations of single cell analysis methods to diagnostic applications in the field of tumour research (especially circulating tumour cells), regenerative medicine, drug discovery and immunology.

Keywords

Imaging, scanning probe microscopy, electron microscopy, Raman, NMR, mass spectrometry, fluorescence, super-resolution microscopy, TIRFM, ~omics (genomic, transcriptomics, proteomics, metabolomics), sequencing, , patch clamp, interaction, signalling, cell type, micromanipulation, trapping, sorting, microfluidics, lab-on-a-chip, AFM, SICM, APT, SECM, FRET, FLIM, FRAP, FCS

Content

I. Introduction: Motivation for single cell analysis

Eukaryotic cells may spend their lives as single individuals; however they have evolved to cooperate closely to form tissues and whole organisms, plants or animals. Conversely, single cell analysis often focuses on individual cells split apart from their tissue of origin. Despite the fact, that this is an artificial situation, single cell investigations gain a special eligibility in raising awareness for small, but substantial differences of single cells in a population of apparently identical cells. Furthermore, single cell investigations play an important role in unravelling mechanistic details of intracellular processes. When we study single cells and try to understand how they function as a biological system we can learn about biological system development, their growth and specialization as well as evolution of single cells and whole organisms. If we can understand biological phenomena at the molecular level, we might be able to understand how subtle differences in cellular phenotype induce biological phenomena such as learning and memory or how cell-specific changes lead to dysfunction and disease states, e.g., in cancer. In order to develop novel therapeutic interventions in response to single cell behaviour, it is important to understand the relationship between biological heterogeneity and signalling pathways. These might involve rare but important events as well as rare cell types, such as stem cells or progenitor cells. Quite often this can lead to a range of questions, for example: 1. what are the important features of a cell? 2. which properties determine the cell type? 3. are the observed differences between two individual cells just the natural cellular variability? 4. are these two similar appearing cells already destined to move in different developmental directions? and 5. how large have the differences between two cells to be in order to assign them to a different cell type? A vast variety of analytical methods and approaches have been developed to study single cells in order to probe their morphological and physiological characteristics, gain molecular, genomic, transcriptomic and quantitative biochemistry information and even monitor their dynamic changes under simulated close-to-*in vivo* conditions. Also, within fully functional organisms, single cells can be identified and tracked which shall ultimately allow studying single cells in their natural, unperturbed environment.

A challenge for single cell studies is the small size of a single cell. Analytical techniques therefore must be able to work with very little sample input. Nevertheless, in order to achieve enough data for significant statistics, they should manage high throughput, and of course – to be affordable in many laboratories – they should not be expensive. In the last few years tremendous advances could be achieved in all fields of single cell analysis. In this review, we

will present the techniques that are currently available for single cell analysis and highlight a few of those recent developments. A wide range of reviews already exists for the different techniques. This review article is not an exhaustive review citing all available references but shall give an overview of the possibilities and maybe create some interest among researchers to try another technique to gain complementary information for their question of interest. Cited review articles and a few original articles shall provide further information for the interested reader. The review is focused on the analysis of mammalian cells, leaving out most of the exciting work done with other eukaryotic cells (such as yeast) or studies on prokaryotic cells. Furthermore, for detailed isolation techniques to retrieve single cells the reader is referred to other recent reviews covering this topic.

II. Overview of technologies and methods

A single cell is a complex system which comprises of several different organelles and components. A schematic of a typical eukaryotic cell from human/animals is shown in Figure 1. When studying single cells a lot of different information from the cell is of interest, e.g., morphology, genotype, the molecular phenotype and various other physiological parameters and metabolic actions.

Morphological characteristics, “how the cell looks like”, can be assessed using a variety of different imaging methods. Many of those methods go way beyond just depicting size and shape of the cell, but can reveal fine structure and even metabolic actions (section III). Imaging methods can be divided into label-free methods which use internal contrast of the sample and contrast-enhanced methods where a specific label, such as a fluorophore or radioactive label is introduced into the cell. Table 1 groups the different imaging techniques according to this criterion.

The oldest label-free technique is optical microscopy (as long as no dyes are involved to stain special organelles, see section III.1). Different types of scanning probe microscopy generate the image by scanning the surface of the cell with a small probe utilizing different sensing mechanisms (see section III.2). Electron (section III.3) and X-ray microscopy (section III.9) reveal the fine structure of cell organisation using electrons or X-rays. Different types of linear and non-linear vibrational spectroscopy (section III.4 and 5) make use of specific photon-matter interactions to generate information-rich images and mass spectrometry (section III.7) creates chemical specific images from the mass information of liberated ions from the sample.

The most prominent example for contrast-enhanced methods is fluorescence microscopy with its various special techniques (see section III.8). However, also in simple optical microscopy the contrast can be enhanced by using special dyes, which e.g. accumulate in the nucleus or other cellular organelles. Nuclear magnetic resonance imaging (section III.6) often utilizes paramagnetic metal particles to enhance contrast. Another criterion how to divide imaging techniques is whether they are destructive or non-destructive. This is especially important if several analysis techniques shall be combined to obtain a more complete picture of the cell of interest (see section III.10).

The widely established omic-technologies, i.e. genomics, transcriptomics, proteomics and metabolomics have tremendously advanced within the last years, making it possible to apply those techniques to the small sample volume contained in a single cell for deeper insight into structure, function, and dynamics on a single cell level (section III & V).

Other physiological parameters, such as ion concentration, mechanical or electrical properties are also essential for a proper function of cellular processes and correct interactions. A vast variety of techniques has been developed to probe those properties and will be discussed in section VI.

Ultimately, all the knowledge and insights gained from single cell analysis will find application in the study of more complex systems, such as tissues or full organs, where the individual cells act in their natural environment in close interaction and cooperation with all the other cells of the same tissue or organ. A few examples of what is already possible now will be given in section VII.

Despite all the advances in single cell research there are still only a few examples of information gained being used in clinical diagnostics. However, it is expected that upon further progress in automation, throughput and general understanding of cellular heterogeneity, several interesting applications in different fields of medicine, such as immunology, regenerative medicine, cancer research or drug discovery, will emerge. (section X)

III. Far more than morphology: Dynamic at the imaging front

Imaging techniques available for the analysis of single cells do not only yield morphological information and answer questions such as "What does the cell really look like?" and "What shape has the nucleus?" but also provide quantitative analysis of chemical species inside subcellular compartments, give molecular details of cellular processes and functions such as growth and death as well as interactions. Imaging techniques aim to detect, identify, visualize and track the spatial distribution of molecules. The interpretation of such changes in structure, organization and activity gives a deeper understanding of cellular processes. Imaging techniques can be divided into direct imaging techniques where intrinsic properties are probed without the need for labels (e.g. Raman spectroscopic imaging) and indirect imaging techniques which achieve higher contrast by the application of labels (e.g. fluorescence microscopy). Imaging probes can be photons, charged particles, such as electrons, atoms as well as sound waves.

In the recent years imaging techniques have been extended and improved to give better resolution, both in space and time. A variety of different methods is now available to study morphology and function, both qualitatively, but also quantitatively [1]. The combination of complementary imaging techniques can greatly amplify available information on structural features and allows the dynamic visualization of morphological changes. [2]

III.1 Optical microscopy – with and without contrast

The oldest method to obtain morphological information from a cell is to investigate it with incoherent white light. From the transmitted and reflected light portions a white light image can be obtained. Such an image depicts reality, however in a filtered, often reduced, and thus imprecise, changed way. It is necessary to be always aware of this limitation. In order to visualize a single cell magnification is needed. Consequently, imaging techniques usually rely on a microscope setup in some way.

Due to its simplicity the most abundant way to get an impression of a cell is to place the culture flask on an inverted microscope and have a look at it. Usually, the cell's shape will uncover immediately if there is a mesenchymal or an epithelial cell present. Often, also the nuclear shape is of interest, e.g. for the differentiation of the leukocyte subtype. Living cells are only thin optical objects and therefore, the contrast is not very high. This can be overcome by staining the sample prior to observation or by using optical contrast methods, such as interferometry (e.g. differential interference contrast (DIC) [3] or quantitative phase imaging. [4]

Phase measurements can be carried out in the full field mode, i.e. providing simultaneously information from the whole image field. This allows for spatial and temporal investigations with time resolution of a few milliseconds. Several label-free, multi-focus imaging methods rely on phase measurements, such as digital holographic microscopy (DHM), Fourier phase microscopy (FPM), diffraction phase microscopy (DPM), spatial light interference microscopy (SLIM), just to name a few. [4]

III.2 High resolution morphology: Electron microscopy (EM)

Transmission electron microscopy (TEM) has become a powerful tool for characterizing structures ranging in size from cells and viruses to small molecular complexes with almost atomic resolution in 3D and to link this information to the macroscopic properties. [5] Information on specifically bound elements like phosphorus, calcium and iron in compartments within sectioned cells can be gained by recording electron energy loss spectra (EELS) at each point of the sample. [6]

Vitrification can preserve biological cells in their near-native, hydrated environment. Automated low dose imaging is necessary to avoid irradiation damage. [7] Cryo-electron microscopy (Cryo-EM) is the method of choice for investigating radiation-sensitive specimens such as single cells. [8-13] In the process the biological sample is studied in a transmission electron microscope under cryogenic conditions. Macromolecular structures inside the cell can be revealed in 3D with a spatial resolution of 4 nm, providing an unprecedented insight into the cellular organization. [7] Cryo-EM can be further sub-divided into cryo-electron tomography, single-particle cryo-electron microscopy and electron crystallography.[5] Hybrid approaches of these techniques and X-ray crystallography as well as nuclear magnetic resonance (NMR) spectroscopy emerge and provide complementary information.

Further sample preparation techniques and instrument modifications for high sensitivity and good contrast as well as post-image processing procedures have been reviewed recently. [7]

In the publicly available repository Electron Microscopy Data Bank (EMDB; http://emdatabank.org/) 3D electron microscopic data of macromolecular complexes and cells can be found with a spatial resolution of 2 to 100 Å.

III.3 Scanning probe microscopy

There exists a vast variety of scanning probe microscopy techniques that all have in common that a physical probe (a kind of “tip”) is moved over the sample surface and records the

surface characteristics. Some of those techniques, such as atomic force microscopy, can achieve a very high spatial resolution. Others, such as electrochemical scanning probe techniques can provide information on mass transfer [14]. Four examples of scanning probe microscopy techniques are presented below.

III.3.1 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) once developed as a high-resolution imaging tool of non-conducting surfaces has become a unique analysis method [15]. AFM overcomes the limited resolution of optical microscopy as well as the limited applicability of electron microscopy. The latter requires vacuum conditions and, therefore, is not appropriate for the investigation of biological samples in their native environments. In contrast, AFM can measure living cells directly in their culture medium without the need of cell fixation or staining. Imaging of cell surface morphology and membrane structure is possible as well as are investigations of dynamic processes involving molecules, organelles and other structures in living cells and interactions at the single-molecule and single cell level [16]. The measurements are based on physical interaction between a nanometer sized tip and the sample. The tip with its contact area of a few square nanometers is attached to the cantilever. This cantilever or the sample is moved in xy-direction via a piezoelectric scanner. A laser beam focused on the end of the cantilever is then reflected onto a photodiode detector. Due to the bending of the cantilever in response to the surface topography the detected laser beam moves and can be detected with pico Newton sensitivity. As a result, a topographic map of the surface is constructed. Generally, a resolution as high as 0.1 nm in lateral and 0.01 nm in vertical direction for molecular or even atomic imaging can be achieved [16]. Due to the fact, that unfixed mammalian cells are very large and soft, the resolution of AFM cell images is limited to approximately 50 nm [16]. Achieving a high spatial resolution down to the single-molecule level on living mammalian cells is still a challenge.

Improvements of the temporal resolution were made by the development of high-speed AFM. This nano-dynamic visualization technique is capable of observing structure dynamics and dynamic processes at a sub-second to sub-100 ms temporal resolution and a 2 nm lateral and a 0.1 nm vertical resolution[17]. The high-speed AFM imaging studies cover a wide range of dynamic molecular processes and structure dynamics, e.g. structure dynamics of proteins in action, self-assembly processes, dynamic protein–protein and protein–DNA interactions, diffusion processes, and molecular processes associated with enzymatic reactions [17].

To overcome the lack of biochemical specificity of the conventional AFM topographic imaging specific antibodies or ligands can be conjugated to the tip enabling the detection and

localization of single molecular recognition sites. Thereby, adhesion force mapping and dynamic recognition force mapping are possible. For details see the review by Hinterdorfer and colleagues [18].

Due to the open AFM setup, combinations with other tools for imaging and functional assays are possible. To select specific components in the heterogeneous cell for AFM characterization, AFM is usually combined with optical microscopy. Fluorescence microscopy is often used for investigations into clinical drugs. Combinations with several advanced optical spectroscopic methods gained impact, e.g. super-resolution imaging and enhanced Raman spectroscopy, allowing for real-time characterization of the molecular composition as well as structural changes. [16]

In the last years rapid progress has been made in AFM imaging of single cells to provide new information on cell surface structure, track the cellular dynamic process, evaluate drug activities and investigate mechanisms of drug action. For detailed information see the reviews by Shi and co-workers [16] and Dufrêne *et al*. [15].

Besides AFM imaging, AFM force mode has overcome some limitations and is capable of gaining quantitative information on cellular interactions at the single-molecule level. Studies concerning sample stiffness and viscoelasticity, cell adhesion, signal transduction and receptor mapping were made [19].

III.3.2 Scanning ion conductance microscopy (SICM)

Scanning ion conductance microscopy (SICM) is another scanning probe technique that can surmount the light diffraction limit and visualize the topography and local changes of living cells.[20] An electrolyted-filled nanopipette scans the sample and the ion current is measured. If the nanopipette is close to the cell surface, a non-conducting surface, the ion flow from the pipette opening is hindered. This relationship between resistance (ion current) and distance can be used to generate a three-dimensional topographical image of the sample surface. Different scanning modes have been developed which differ in speed and surface roughness that can be captured by this technique without destroying the probe [20]. The spatial resolution depends on the opening diameter of the nanopipette (resolution is roughly 3/2 of the diameter [21]) and values as low as 5 nm have been reported. Detailed three-dimensional SICM images enabled investigation of the morphological response of living cells on different stimuli, such as cytokines, corticosteroids, nanoparticles, shear stress or even surface changes after stimulated exocytosis. From the 3D image of the cell also information on the cell volume and volume changes can be gained. [20] The electrical distance control in SICM avoids mechanical damage of very soft and delicate samples such as living cells and unlike with AFM, it is

possible to image very fine and lose structures of cells, such as cell extensions, without any distortion. [22]

SICM can be combined with various other detection methods and act as a multifunctional tool: combination was reported with optical methods such as scanning near field techniques, fluorescence microscopy and even FRET; as well as with electrochemical recordings, such as patch clamp and ion-selective microelectrodes. Single-molecule fluorescence microscopy and SICM can reveal information on protein functions [20] as well as insights into cell membrane organization and function. [23] Combination of SICM and FRET can be used to elucidate pathophysiological mechanisms as was done to gain insights into the role of the redistribution of the β2-adrenergic receptor in heart failure [24] Combination with patch clamp with two glass pipettes can be used to generate functional maps of ion channels. [25] When the same pipette is used to first scan the cell surface with high topological resolution, and then for ion channel recording it is called "smart patch clamp" as the nanodomain for creating the seal with the surface can be chosen with high precision. [26]

The scanning tip can further be utilized to deposit molecules onto the surface or modify the local ion concentration. Such biochemical applications of SICM have been reviewed elsewhere. [27, 28]

III.3.3 Atom probe tomography (APT)

Atom probe tomography (APT) provides three-dimensional maps of ion compositions and was classically used to map metals and semiconductors with a sub-nanometer resolution. Recently, Narayan *et al*. were able to record cellular ions and metabolites from unstained, freeze-dried mammalian cells. Thereby, the reconstructions of cellular sub-volumes at high resolution revealed a surprising amount of spatial heterogeneity of specific chemical species within the cell [29]. APT is based on the field evaporation. Thereby, ions are desorbed from a needle-shaped sample by application of a very intense electrical field of several volts per nanometer under vacuum and at cryogenic temperatures, followed by the detection with a single-ion sensitive detector. The combination of APT with time-of-flight measurements allows the chemical identification of these detected ions in a mass spectrum.

III.3.4 Scanning electrochemical microscopy (SECM)

Scanning electrochemical microscopy (SECM) is suitable to monitor the electrochemical events on or in close proximity to a surface. Although, SECM cannot provide the same topographical resolution as atomic force microscopy or scanning electron microscopy, it is a

powerful analytical tool for the imaging of surface topography and the mapping of electrochemical activity of living cells on a sub-µm scale [30].

The key element of SECM is a small scaled electrode serving as a mobile probe and recording changes in electrochemical potential. This so-called ultramicroelectrode is characterized by its enormous sensitivity (enabling the detection of even trace amounts of analytes), its short response time and the high spatial resolution. It consists of an electroactive material (e.g., carbon, platinum, gold) and can have different shapes (e.g. disks, rings, bands, cylinders, spheres, hemispheres) depending on the application. Due to the electron transfer reactions of the detected analytes at well-defined potentials the electrochemical detection is highly specific. The small size of the electrode, which ranges from micrometers to nanometers, allows for an unhampered approach to cells and provides high lateral resolution for imaging [31].

Different imaging modes can be used, e.g. constant-height and constant-current mode. Constant-height mode imaging is the traditional scanning mode where the distance between the tip and the sample is stable and the tip scans across the surface in the xy plane. However, a feedback-based guidance system is required to maintain this distance. In the constant-current mode the device attempts to maintain a constant current by changing the substrate to tip distance. For more details see the review by Bergner *et al*. [30]

The use of ultramicroelectrodes to study single cells requires appropriate dimensions, stability during the whole experiment, high analyte sensitivity and selectivity and a high signal-to-noise ratio. Furthermore, biomolecules from the cell culture medium or even cells themselves can bind irreversibly to the electrode and consequently reduce the sensitivity. These requirements and handling problems still hold potentials for improvements.

SECM offers several advantages compared to other methods: (1) in contrast to fluorescence microscopy (section III.8) no staining or labelling is required and (2) unlike the AFM probe, the SECM probe does not need to touch the cell, thus time-lapse measurements without mechanically scratching the cell are possible [30, 32]. Nevertheless, most SECM imaging experiments were conducted with the addition of a certain redox mediator which is usually non-physiologic and undesired. However, Zhang and co-workers introduced dissolved oxygen as the redox mediator in the medium solution which provides an opportunity of label-free imaging of cellular morphology. [32] Quantifying the flux of molecules entering or leaving a cell, studying ion transport in channels, probing the local electrochemical reactions at and inside living cells are possible application fields of SECM. Furthermore, membrane permeability and the presence of metabolites can be detected and enzymatic activities can be

evaluated [31, 33]. Zhao and colleagues successfully detected reactive oxygen species (ROS) released from living macrophages [34] as well as single human bladder and kidney epithelial cells [35] by means of SECM. Real-time filming of the movement and morphological changes of living cells was performed via time-lapse SECM [32]. For a detailed insight into the application of SECM see the following reviews [30, 31, 33].

In addition, simultaneous investigations of (living) samples are possible by combining SECM with other biosensing techniques. Microfabrication techniques hold promise in supporting SECM-based investigation by providing fluidic-based culture platforms that can control cell environments at well-defined length scales. Integration of an inverted fluorescence microscope allows simultaneous or subsequent evaluation of the state of the cells following SECM measurements [31].

SECM in combination with optical microscopy is a powerful analytical tool to receive multidimensional information on complicated cellular processes. Therefor, integration into optical fibre probes, near-field optical microscopes, atomic force microscopes and confocal (laser) microscopes is carried out. The construction of a smaller optical fibre probe will enable electrochemical and optical imaging with nm-resolution [33].

III.4 Raman spectroscopic imaging

Raman spectroscopic imaging is a hyperspectral imaging technique that can provide information-rich chemical images in a non-invasive and non-destructive manner. Raw data of Raman spectral images include spatial xy-information as well as a spectral dimension which gives the vibrational signature of the overall molecular composition, e.g. from proteins, nucleic acids, lipids, carbohydrates and inorganic crystals. Addition of a spatial z-component is also possible. Spectra from different points of an image can be acquired in point, line or map mode depending on the applied optics and the capacity of the detector, which allows for different temporal resolution. The Raman effect is based on inelastic photon scattering of incoming monochromatic laser light on molecular vibrations. As almost all molecules are Raman active, no external label is required. However, since Raman scattering is a very rare event, detection of Raman spectra requires highly sensitive detectors. Although the technique had long been applied in chemical research, it was introduced only in 1990 for studies on single cells.[36] Raman spectra from mixed samples such as biological cells are very complex and require statistical analysis for meaningful interpretation. Up to now, Raman spectroscopy is an uncommon method in cell biology, but slowly entering the field. Improvements in detector technology [37] and data evaluation procedures led to some interesting Raman-based studies during the last decade which should further increase its relevance in single cell

research in future. Raman micro-spectroscopy could depict cell morphology and compartments such as nucleus, cytoplasm and vesicles.[38] Cytochrome dynamics have been studied during apoptosis.[39] Lipid droplet dynamics and composition were addressed in hepatocytes[40] as well as hepatic stellate cells[41]. With the help of Raman spectroscopy is possible to distinguish different cell types and build classification models which can assign the cell type based on the spectral characteristics [42-45]. For such single cells classification models, it is important to perform proper sample size planning and take random testing uncertainty into account. [46] Furthermore, Raman spectroscopy was used to identify pathological states of the cells, follow differentiation, transformation and viability as well as provide insights into metabolism and chemical reactions [42, 47, 48]. Also single cells in the tissue surrounding could be identified by means of Raman spectroscopy in combination with statistical analysis methods [49].

In combination with microfluidic Raman spectroscopy can be used to sort individual cells (**Raman activated cell sorting, RACS**). [50, 51] However, the achieved throughput is by far less than for fluorescence activated cell sorting (FACS) (see also section VIII.3).

One reason for that is the inherently weak Raman scattering efficiency. Several methods have been developed to increase Raman signal intensity. In **resonance Raman spectroscopy** the wavelength of the Raman excitation light matches with an electronic transition of the molecules of interests. The Raman bands of the vibrations that couple to that electronic transition experience an increase in intensity by 4-6 orders of magnitude. [50] Resonance Raman spectroscopy was used to study hemoproteins and monitor oxygenation state of red blood cells. [52] Furthermore, the technique was utilized to follow beta caroteine loaded nanoparticle uptake in living cells as possible drug carriers [53] as well as to follow differences in internalization via patch clamp between free haemin and peptide capped haemin into living HEK cells. [54]

Another method which can increase the observed Raman signal intensity is **surface-enhanced Raman spectroscopy (SERS)**. In the close vicinity to a rough metal surface (mostly gold and silver are used), an increase in intensity of up to 14 orders of magnitude is reported. [50] The used nanoparticles play a crucial role. [55] They can be functionalized and their uptake by single cells can be tracked.[56] Surface-enhanced Raman spectroscopy has been used to detect cancer cells and investigate treatment efficiency. [3]. Multiplexed optical sensing using SERS was reviewed by Rodriguez-Lorenzo *et al*. [57]

If the SERS-active particle is reduced to a single tip and combined with an AFM (see section III.2.1) one can speak of **tip-enhanced Raman spectroscopy (TERS)**. By means of TERS

the surfaces of single cells can be investigated with a high spatial resolution (down to 10-20 nm) [58]

The complementary vibrational spectroscopic imaging technique, **IR absorption spectroscopy**, uses light in the mid IR region to characterize the sample. The diffraction limited resolution is on the order of a few micrometers, the same size as single cells. [59] Therefore, IR spectroscopy is mainly used to study large cells, such as skin fibroblasts, giant sarcoma cells [60] and oral mucosa cells [61]. Using single spot measurements from individual cells it is possible to follow cell-cycle dependent variations [62] and conformational changes in DNA [63]. Furthermore, IR spectroscopy is successfully applied to identify single cells in tissue slices [64].

III.5 Nonlinear optical imaging

A thousand-fold improvement in the Raman signal intensity can be reached by generating **coherent anti-Stokes Raman scattering (CARS)**. Thereby, a target molecule is irradiated via two short-pulse laser beams (pump and Stokes beam). The frequencies of these beams must be tuned so that the frequency difference corresponds to a vibration of the target. In that case, coherently vibrating molecules in the sample volume will scatter the probe beam, resulting in a coherent signal [65, 66] . This multiphoton process offers intrinsic three-dimensional sub-µm resolution and an image contrast, which is obtained from inelastically scattered light by the vibrations of endogenous chemical bonds [67]

CARS is suitable to examine live cell dynamics with its high chemical specificity and its label-free and non-invasive character. However, the complexity and the high costs of the laser systems prevented a widespread application of CARS microscopy so far.

The major asset of CARS is the delivery of high signal intensity from lipid C-H bond stretches. Therefore, the majority of CARS applications involve lipid imaging and lipid quantification which have been real challenges in analytics before. This is because lipid-specific markers for fluorescence microscopy are difficult to produce and the labelling process often affects lipid localization and function. [67] Although, at first glance, the analytical focus of CARS microscopy on lipids may seem like a limitation, the ability to image them has provided valuable insight into a numerous diversity of biological processes in which lipids play an important role, e.g., in biological membranes, as energy storage molecules or as messengers in cell communication. [43, 65] Lipid vesicles inside HeLa cells, membranes of lysed erythrocyte cells, the growth of lipid droplets in live adipocyte cells, the organelle transport in

living cells as well as lipid storage in the nematode *Caenorhabditis elegans* have been readily visualized by CARS microscopy. The composition and packing of individual cellular lipid droplets has also been imaged using multiplex CARS microscopy. For more details see the review by Krafft *et al.* [43]

In addition, by tuning the CARS laser into amide I vibration, protein distributions in epithelial cells were visualized. Using the OH-stretching vibration of water, cellular hydrodynamics have been investigated with sub-second time resolution. The C-D stretching vibration also gives raise to a strong signal. Therefore, deuterium labels are used in many CARS experiments. [43] Furthermore, monitoring of dynamic cellular processes, such as lipid metabolism and storage, organelle movements, tracking of molecules within cells and imaging of exogenously added probes or drugs have been successfully accomplished. [65]

The CARS setup can be combined with other methods, e.g. microfluidic devices (CARS flow cytometry) [43] or two-photon fluorescence microscopy. These multimodal instruments pave the way for further applications. [67]

Stimulated Raman scattering (SRS) is another label-free imaging method, which overcomes some limitations of CARS microscopy. It is characterized by an energy difference between pump and Stokes photons, which is resonant to the vibrational frequency of a special chemical bond in the molecule. Following, non-linear interaction the excitation of that molecular vibration is stimulated. This is accompanied by energy transfer from the pump beam to the Stokes beam resulting in the intensity loss of the pump beam and the intensity gain of the Stokes beam. In case that the energy difference between the two laser beams does not match with the target molecule vibrational frequency, a non-resonant background appears. [68]

SRS allows imaging of biological molecules in living cells with a high resolution, sensitivity and speed. Lipid measurement, drug delivery monitoring and tumour cell detection are only some of the application fields of SRS. [68] This year Wei and colleagues demonstrated for the first time imaging of newly synthesized proteins in live mammalian cells with high spatial–temporal resolution via SRS combined with metabolic incorporation of deuterium-labelled amino acids. Importantly, this method can readily generate spatial maps of the quantitative ratio between new and total proteomes.[69]

Second harmonic imaging microscopy of biological specimens exploits a nonlinear optical effect known as **second harmonic generation (SHG)**, where the energy of the incident photons is scattered via a process of harmonic upconversion, instead of being absorbed by a molecule.[70] In detail, an intense laser beam passes through a polarisable material with a non-centrosymmetric molecular organization. A nonlinear mixing of the excitation light then

results in the generation of a wave at twice the optical frequency.[67] SHG strongly depends on the polarization state of the laser light and the orientation of the dipole moment in the molecules that interact with the light.[71] It is a label-free technique, which is typically used to detect collagen in the extracellular matrix. A major disadvantage of SHG microscopy is that the signal from cellular components is generally weak. However, second harmonic generation imaging can reveal the non-centrosymmetric and inhomogeneous structure of the object.[71] Reshak and co-authors detected strong SHG from the granal regions in the starch free chloroplasts of single cells. Upon illumination the chloroplasts changed their orientation, which affected the SHG signal.[71] So far, only a few studies of SHG on single eukaryotic cells exist.

III.6 Nuclear magnetic resonance imaging (NMRI)

Nuclear magnetic resonance imaging (NMRI) represents a further non-invasive imaging method. This technique is based on the molecular fingerprint resulting from the chemical shifts of resonance frequencies of nuclear spins within a strong magnetic field. [72] Therefore, NMRI is well suited to follow nanoparticles for cell localization in organs and tissue. Specific molecular probes that concentrate on target cells after their injection into living organisms can be localized via targeted NMRI, e.g., antibodies linked to ultra-small super-paramagnetic iron oxide nanoparticles that bind to tumour cells. [73, 74] The first NMR microimages of single cells were obtained from frog ova with a spatial resolutions in the order of magnitude of 10 μm [75] due to a combination of small-diameter radio frequency coils and high magnetic field. In 2000 Grant and colleagues demonstrated for the first time the feasibility of NMR spectroscopy to localize osmolytes and metabolites within single neural cells from the sea hare *Aplysia californica*[76]. Lee *et al.* acquired compartment-specific spectra of an oocyte from *Xenopus laevis* and monitored the uptake kinetics of an externally applied drug into the individual subcellular compartments *in vivo*[77]. Technical improvements, such as small-volume NMR probes, and an accompanying enhancement of the detection limit enables the investigation of samples with the sizes of typical eukaryotic cells. [73]

III.7 Mass spectrometry imaging (MSI)

Another label free method for successful chemical characterization is mass spectrometry imaging (MSI), which is well suited to image and profile individual cells and subcellular structures. Similar to other imaging techniques, e.g. Raman mapping (section III.4), one spectrum is acquired at each point of a spatially defined grid. In MSI this spectrum contains

mass information from that particular spot. Ion images showing the distribution of the selected peak can be generated by applying mass filters to the collected mass spectra.[2]

MSI offers several advantages compared to other imaging techniques: (1) it has a high chemical specificity; (2) no pre-selection or even knowledge of the analytes is necessary prior to the analysis and, (3) the number of co-registered ion images is, in theory, only limited by the number of distinct detected ions and the resolution of the spectra. [78] Nevertheless, mass spectrometry is an invasive method necessitating further advances in sample preparation and data analysis as well as sensitivity improvement for cell-scale experiments.

For an extensive review concerning mass spectrometry imaging of single cells see the one of Lanni and co-authors[78] or the one of Masyuko *et al*.[2]

To answer the broad range of analytical as well as biological and biomedical questions secondary ion mass spectrometry (SIMS) and matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) are the two most established mass spectrometry techniques to obtain morphology related chemical information from single cells.[79] SIMS is a surface analysis method providing chemical information from the first few nanometers of the sample surface. SIMS can routinely reach the highest spatial resolution at the sub-micron level (< 50 nm) in comparison to other MSI techniques. In addition, it delivers useful quantitative information. [80] Thereby, the detectable ions are limited to a small mass range of a few hundred Dalton. With this technique it is possible to localize analytes in two- as well as three dimensions within single cells. [78]

SIMS can be divided into dynamic and static SIMS. Dynamic SIMS is usually combined with other high-resolution imaging techniques, e.g. electron-, atomic force- and fluorescence microscopy.[78] High-resolution mapping of endogenous and exogenous ions and molecules provides unique insights into single cells: the localization of proteins and nucleic acids by detecting elemental sulphur and phosphorus is possible[81] as well as the localization of iron in diseased cells (which found applications in Alzheimer research) [82, 83]. Due to the detection of endogenous inorganic ions, e.g., Na^+ and Cl^-, information on the physiological state of the cell including mitosis, membrane potential and transport can be derived. [84] Furthermore, localization[85] and evaluation[86] of the efficiency of pharmaceuticals as well as direct cancer detection[87] are possible. For detailed insights into the applications and capabilities of dynamic SIMS the interested reader is referred to the article by Chandra in "The Encyclopedia of Mass Spectrometry" [84].

Static SIMS (also known as time-of-flight (TOF) - SIMS) is conveniently combined with a TOF analyzer acquiring full mass spectra for each pixel. Static SIMS is characterized by an

undamaged surface after measurement due to a limitation of the primary ion fluence of less than 10^{13} ions per cm^2. Due to this static limit, the application to cellular imaging is confined to membrane-localized molecules including membrane phospholipids[88] and other small molecules, e.g. cholesterol and vitamin E[78].

One of the most important efforts was achieved by Steinhauser and colleagues, who developed multi-isotope imaging mass spectrometry. This SIMS-based technique applied to individual cells from diverse organisms (Drosophila, mice, humans) allowed the measurement of stable isotope-containing functional tracers. Subcellular spatial resolution and quantitative information are among to the methodological achievements of this work. [89]

Matrix assisted laser desorption ionization (MALDI) MS is well-established for tissue-based studies, but has become routine for subcellular investigations. Usually, ultraviolet lasers are used for laser desorption ionization. [2] MALDI MS is the most versatile and easy-to-use MSI technology in order to identify the molecular signature of pathological phenomena [90]. This technique offers a large mass range, a high sensitivity for detection of analytes and is also suitable to investigate complex mixtures. Thereby, the sample preparation is easy, although the lack of appropriate matrix application methods, which limits the resolution of imaging, leaves room for improvements[91-94]. For MALDI MS measurements the sample is embedded in a matrix of organic substance crystals or liquid crystals and scanned by a focused, pulsed or continuous laser beam. The analyte molecules are (1) desorbed due to the energy absorption by the matrix, (2) ionized, (3) extracted from the source, (4) analyzed by their mass-to-charge ratio, and finally (5) detected. Thereby, a localization of metabolites, proteins, peptides and lipids as well as DNA and RNA is possible. MALDI MSI was successfully applied in single cell and organelle profiling studies (reviewed by Lanni *et al.*[78]). Improvement of the spatial resolution for cellular and sub-cellular investigations was one the main challenges in the last few years. Optimizations in the instrumental setup of MALDI MSI achieved a routine working resolution between 4 and 7 µm. [95-98] Spengler and Hubert were able to reach an imaging resolution between 0.6 and 1.5 µm via SMALDI (Scanning microprobe MALDI).[99] The capability of SMALDI was shown by imaging human renal carcinoma cells with a resolution of 2 µm and detecting masses up to 5 kDa[100]. Combination of MALDI MSI with other methods, e.g., Raman spectroscopic imaging, infrared imaging or Fourier transform ion cyclotron resonance MS, would truly benefit MALDI MSI informative value [90]. Also, combination of other MS-based techniques, e.g., matrix-free laser desorption/ionization MS, with fluorescence and Raman microspectroscopy gains relevance, as it was shown by a multidimensional chemical analysis of *Euglena gracilis* and *Chlamydomonas reinhardtii*.[101]

Furthermore, three-dimensional cross-sectional images of rat brain and plant tissues could be constructed by laser ablation electrospray ionization (LAESI) MS combining two-dimensional lateral imaging with depth profiling. Shrestha *et al*. demonstrated in situ cell-by-cell imaging of plant tissues. Chemical imaging of the metabolite cyaniding (purple pigmentation in onion epidermal cells) using single cells as voxels reflects the spatial distribution of biochemical differences within a tissue.[102]

In addition, scanning near-field optical microscopy (SNOM) MS, laser ablation-inductively coupled plasma (LA-ICP) MS and nanostructure-initiator mass spectrometry (NIMS) have a high potential as future methods for subcellular investigations since they work under atmospheric conditions and are therefore capable to image living cells[78].

III.8 Fluorescence microscopic imaging

Fluorescence microscopic imaging is a commonly used approach to visualize cells and organelles, and to study intracellular interactions. A wide range of organic fluorophores and fluorescent proteins is available that can be selectively inserted into a cell or even expressed therein. During the last years also luminescent heavy metal complexes [103] and quantum dots [104] were made biocompatible to be used in live cell imaging. Quantum dots have the advantages of being chemical and photo-resistant reporters with a narrow and tuneable emission ranging from UV to NIR. [104] They could even be used for single biomolecule tracking inside living cells. [105]

Two main challenges are faced in the field of fluorescence-based microscopy. On the one hand high spatial resolution is of importance if nearby molecules inside or on top of the cell shall be separated. On the other hand temporal resolution is limiting if dynamic events in living cells are of interest. An essay emphasizing critical aspects for live cell imaging was published recently by Sung[106].

III.8.1 Confocal Laser Scanning Microscopy (CLSM)

To achieve spatial resolution in z-direction confocal laser scanning microscopes either equipped with a single pinhole or a scanning disk for faster data acquisition are still widely used. The principle of confocality is based on the conjugation of the sample plane, where incoming light from the objective is focused and a pinhole in its optical plane, where emitted light from the sample plane arrives. The review by Stehbens *et al*. [107] describes recent developments of confocal imaging setups.

An intriguing recent application is the detailed observation of lamina associated domains (LADs), the missing of their heritability and their spread after mitosis. LADs are sites of

contact between DNA and nuclear lamina. Imaging is based on enzymatic adenine-6-methylation in DNA in case of its contact with the lamina and subsequent fluorescence-labelling by the fragment of another enzyme. The modifications work as an event memory. Both active proteins are only expressed after transfection. While the authors state randomness of LAD establishment, the study also points to possible influences of LADs in gene regulation.[108] This represents another hint for the plasticity the cell type concept has to fulfill. Others concentrated e. g. on the elucidation of the moment of nuclear pore formation during reestablishment of the nuclear envelope. Evidence was found that all components join in the nascent envelope and therefore, against the assembly or maintenance of a prepore complex at endoplasmatic reticulum membranes.[109]

Also in the field of cytoskeletal research confocal LSM still reveals valuable information. Dunsch and collegues e.g. reported on spindle orientation during mitosis depending on a special dynein light chain[110].

III.8.2 Revealing of molecular interactions: FRET and FLIM

Fluorescence or **Förster resonance energy transfer (FRET)** experiments have been applied to study inter- or intramolecular interactions since decades [111, 112]. For a recent review read e.g., the one by Zadran *et al.*[113]. FRET exploits a radiationless process transferring energy collected via excitation from an electronic ground state to the first excited state by a first fluorophore, called the donor, to a second fluorophore, called the acceptor, which emits a photon while relaxing back to its electronic ground state. The photon emitted by the acceptor is red shifted compared to the photon that would have been emitted by the donor without the occurrence of FRET. Donor and acceptor constitute a FRET pair. There are several requirements to be fulfilled for FRET to happen. First of all, the emission spectrum of FRET-donor and the excitation spectrum of FRET-acceptor have to overlap. There are several FRET pairs commercially available. For a list of widely used fluorescent proteins, represented also in FRET pairs see Wiedenmann *et al.*[114]. In addition, the orientation of the transition dipole moments of the FRET pair influences FRET efficiency. Illustrative DFT calculations concerning several FRET pairs where published by Ansbacher and colleagues[115]. Finally, the characteristic making FRET a valuable tool for interaction studies is its distance sensitivity. Since the efficiency of FRET decreases proportional to the sixth power of the distance between donor and acceptor, FRET is known to occur only within a molecule distance of 1 to 10 nm. Nowadays, FRET is applied to whole cells to study e.g. HER2-EGFR dimerization[116] or spatio-temporal kinase activity[117]. Cyan fluorescent protein (CFP) – yellow fluorescent

protein (YFP) FRET tools were presented furthermore, to sense cellular energy state via adenosine triphosphate (ATP) recognition.[118, 119] Notably, ATP action as an energy transition quencher is disputed by a more recent work assigning the ATP effect rather to interaction of ATP and the FRET-donor.[120] The adaptability of FRET experiments is still limited due to strict spectral requirements for fluorophore combinations challenging the synchronous detection of parallel events in the cell. Methods like compartmentalization or the coupling of one donor to different acceptors to overcome these confinements are discussed in a recent review by Depry *et al*.[121]. Geißler and co-workers even described the possibility to distinguish acceptor emissions of five different FRET acceptors in one experiment by employment of a single donor (Tb complex) for energy transfer and advanced spectral crosstalk correction, although not in single cells but in serum.[122] Ouyang and colleagues were able to study Src kinase and MT1-MMP activation simultaneously upon stimulation in living HeLa cells using two completely different FRET pairs.[117]

FRET enables not only the detection of the fluorescence of an acceptor fluorophore that has not excited by the incident laser beam but also shortens the time the donor is in the excited state. This results in a decrease of its fluorescence life time. Thus, interaction can also be concluded from changes in donor fluorescence life time when an acceptor is e.g. coupled to an enzyme substrate or a receptor ligand. Studies of intra-molecular changes in living cells are also possible. As an example, the epidermal growth factor receptor conformations in free and ligand-bound form were examined only recently to address its ability of self-inhibition.[123]

The fluorescence lifetime is sensitive to changes in the molecular environment. Thus, information on ion concentrations, pH-values or oxygen presence can be derived from a change in fluorescence lifetime. The possibility to distinguish several fractions of a certain fluorophore inside one cell makes **fluorescene lifetime imaging microscopy (FLIM)** especially advantageous. [124] For a detailed description of FLIM and further exemplary applications in living cells see Sun *et al*.[125]. For an overview of different FLIM techniques and their microscope implementation see Becker. [124]

III.8.3 Following motion inside the cell: FRAP and FCS

Fluorescence recovery after photobleaching (FRAP) allows time resolved observation of molecule dynamics. It requires the molecule of interest to be tagged or fused to a fluorophore and the photobleaching with intense (laser) light of a region of interest. After the bleaching event, reoccurrence of fluorescence in that area is detected. Furthermore, it is possible to flip the experiment around by bleaching an area adjacent to the observed field and to follow

fluorescence loss postbleaching. For a review covering the fundamentals of FRAP see the one by Reits and Neefjes[126]. For a critical, more recent review with focus on nuclear protein dynamics the one by Mueller *et al.*[127] is recommended. Recently, FRAP e.g. served to visualize H-Ras exchange in free diffusion between B- and T-lymphocytes via membranes of tunnelling nanotubes[128] or heat shock factor 1 dynamics[129]. Advancement in measurement precision by combining FRAP and **fluorescence correlation spectroscopy (FCS)** was described by Im and colleagues[130]. Both techniques complement each other with respect to sensitivity and speed of the detectable reaction kinetics.[129, 130]

In brief, for FCS a defined volume, the focal volume, is laser illuminated and the resulting fluorescence is detected continuously. Fluctuations in the fluorescence read out over time allow for the calculation of the diffusion coefficient of the fluorescing particle. Binding events and dissociation constants can be concluded from FCS measurements, in addition. For an exhaustive description of the FCS principle the review of Elson is recommended [131]. FCS is beneficial for single cell application, as is demonstrated by a number of FCS-based studies on molecule mobility, e.g. on nuclear receptor DNA binding[132] or signal transducer activity in cellular stress response[129] as well as on apoptosis onset based on caspase activity assessment[133, 134]. A dual-colour version of FCS is **fluorescence cross-correlation spectroscopy (FCCS)**. For FCCS measurements two spectrally distinct fluorophores are required. Diffusion dynamics are measured for each of the fluorophores as in conservative FCS. However, the results are cross-correlated afterwards, thus, revealing simultaneous or independent movements. The fluorophores can ,e.g., be situated at two members of a protein complex or both on one target molecule of an enzyme on the different sides of the restriction site. Transcription factor association[135] or enzyme activities[136, 137] were studied that way. For a review on FCCS, dealing also with further examples, see the one by Bacia *et al.*[138]

III.8.4 Super-resolution fluorescence microscopy

Confocal laser scanning microscopy is limited in lateral and axial resolution by diffraction. However, quite often it is of interest to observe also smaller structural detail and features in a cell. Several microscopy techniques evolved to gain resolution beyond the diffraction limit. Stimulated emission depletion (STED), structured illumination microscopy (SIM), photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are famous representatives. For a generalized application oriented review the ones by Ball *et al.*[139] and Schermelleh *et al.* [140] are suggested.

Spatially addressed modulation:

Stimulated emission depletion (STED)

In STED de-excitation of fluorophores is exploited by de-exciting a ring-shaped area using stimulated emission and detecting remaining spontaneous fluorescence in the avoided central space afterwards. A lateral resolution down to 50 nm was reported by Jans *et al*. [141]. However, due to point by point scanning and fluorescence detection the acquisition of a STED image is time consuming.[142] The panel of applicable fluorophores is limited as well.[142] A related methodology exploits reversible saturable optically linear fluorescence transitions (RESOLFT) using special photo-switchable fluorophores which can be kept in the non-fluorescent state for prolonged time, thus allowing for lower laser intensity for the on to off-switch. [3]. STED was applied to study neuronal architecture events on synapses.[143, 144] Distribution of TOM20, a component of the translocase of mitochondrial outer membrane (TOM) complex, was addressed by Wurm and colleagues. They found it clustered and in varying quantity depending on cell position in a colony.[145] In a recent contribution by Jans and co-workers the resolution capacity of STED served to study the organization of MINOS complexes in mitochondria. The authors report on highly ordered mitofilin, MINOS1 and $CHCHD_3$ structures.[141] TOM20 and MINOS abundance differences with respect to mitochondrial nuclear distance were uncovered in either study.[141, 145] For a review concerning STED and further high resolution microscopy techniques see Tønnesen & Nägerl[146] or Lidtke & Lidtke[147].

Structured illumination microscopy (SIM)

Another approach to improve the spatial resolution in light microscopy is pursued by SIM. For this full-field fluorescence method structured excitation light, exhibiting spatially varying intensity is used instead of homogenous illumination. Multiplicative meeting of incoming fine structured light with the fine structural details of the sample leads to specific patterns of emitted fluorescence, called Moiré fringes. Back calculation of the originally light emitting sources, meaning sample structure, from the measured data is possible as far as enough images under different illumination conditions are available. The required number of images depends on the pattern of the excitation beam. Each image has to be acquired with a different phase of the excitation light.[148] A two fold increase in resolution compared to wide-field imaging was simultaneously achieved in lateral and axial dimension.[140, 148] A resolution of 50 nm in a fluorescent bead sample has been shown for saturated SIM which involves a non-linear modulation pattern.[149, 150] For a review of super-resolution microscopy methods

containing especially informed knowledge on SIM see the one by Schermelleh, Heintzmann and Leonhardt.[140] 3D-SIM led to unprecedented accuracy in replication foci quantification, revealing a three to five fold higher number than CLSM-based counting and a decrease in amount during cell cycle S phase.[151] Moreover, stratification between space occupied by nuclear pore complexes, nuclear lamina and chromatin was visualized impressively, pointing furthermore to a strict separation of nuclear pores and chromatin.[152] In addition, perfect matching of 3D-SIM and fluorescence in situ hybridization has been shown by Markaki and co-workers who report on diverse examples concerning the spatial extend of heterochromatin interaction in interphase.[153]

Stochastic modulation: Point-localization super-resolution microscopy

The term point-localization super-resolution microscopy or pointillism – as suggested by Lidke *et al*. [154]- comprises amongst others PALM and STORM. The techniques share the strategy of repeated stochastic fluorophore activation followed by wide field image acquisition and calculation of exact fluorophore positions from a set of such images acquired from the same field but with stochastically varying fluorophores in the "on" state. These calculations are based on precise fits of detected single molecule emissions. As a review focusing on point-localization super resolution microscopy strategies and their background that also contains application examples the one of Sengupta *et al*. [155] is recommended.

A derivative method introduced by Dertinger *et al*.[156] is super-resolution optical fluctuation imaging (SOFI) which relies on flickering fluorophores. For SOFI a number of images have to be acquired as well. In SOFI higher-order fluctuation statistics is exploited without the need to localize individual molecules. A simple variance projection is an example of a SOFI image of order two. SOFI was already shown to improve resolution and contrast and to suppress background in living HeLa cells also in a two-colour version.[157] SOFI typically works with relatively dense concentrations of simultaneous emitters, but yields only moderate resolution (100nm typically). Accepting the confinement of several possible solutions for molecular assignments in dense data led to the development of 3B (Bayesian analysis of blinking and bleaching) which enables live cell imaging of podosomes at 0.5 Hertz with a resolution of around 40nm [158]. Using the example of those small podosomes (diameter 0.5-2µm), which sit like small "adhesive bowls" on the outer membrane of macrophages, Figure 2 illustrates the spatial resolution power of a few of the introduced high resolution approaches compared to widefield microscopy and confocal laser scanning microscopy.

III.8.5 Hyperspectral fluorescence imaging

While the techniques described above are suitable to achieve a spatial resolution down to 50 nm (lateral resolution achieved by STED)[141], they suffer from time consuming pointwise fluorescence detection (CLSM, STED) and the necessity of repeated image acquisition (SIM, PALM, STORM, SOFI), respectively. If different fluorophores are of relevance, acquisition time raises accordingly and in addition, due to the necessity of mechanical configuration switches. Acquisition of hyperspectral image maps overcomes this problem. Hyperspectral means the detection of spectral regions covering emission ranges of different fluorophores on a CCD (charge coupled device) at ones instead of a narrow part to avoid overlap of emission. Information about the presence of a certain fluorophore at a point has to be derived from deconvolution afterwards. Uhr and co-workers employed a hyperspectral imaging device for quantitative assessment of 10 independent markers in individual breast tumour cells from solid tumour tissue as well as circulating tumour cells. A tumour molecular signature for solid tumour is stated.[159] An image mapping spectrometer for fluorescence measurements avoiding the scanning obligation was described by Gao and colleagues[160]. It enables to acquire maps with 285 x 285 pixels at a time rate of 7,5 frames per second (fps). Simultaneously, the full spectral information (60 channels with an average sampling interval of 3.3 nm) is projected from each sampled pixel position onto a large format CCD detector. Thus, data acquisition at a single moment in time results in a 3D matrix (data cube) containing x- y- and λ-information.[160] In a recent contribution Elliott and colleagues report the use of an adapted version to study intracellular cAMP and Ca^{2+} concentration dynamics simultaneously in living representatives of a pancreatic β-cell line at 2 fps upon glucose stimulation. They reveal anti-correlation of the concentration oscillations of these signal transducers.[161] As an additional advantage, Leavesley and colleagues state the superiority of hyperspectral fluorescence detection compared to the narrow band mode with respect to sensitivity and specificity of GFP positivity determination of single cells against highly fluorescent background in pulmonary tissue.[162]

III.8.6 Total Internal Reflection Fluorescence Microscopy (TIRFM)

A possibility to study the distribution of molecules in- and outside the cell body is total internal reflection fluorescence microscopy (TIRFM). It is based on light refraction at the interface of media with unequal optical density. If the incident beam passes from the medium with higher refractive index to the one with lower refractive index, there is a certain critical angle depending on these refractive indices from which any light can pass [3]. Nevertheless, a

non-propagating electromagnetic field exists, the evanescent wave, which excites fluorophores close to the phase border. Since the field strength decreases rapidly with the distance to this border excitation within the field is highly selective avoiding out of plane fluorescence reliably.[3, 163] Several of the afore described super-resolution microscopy techniques are applied preferentially in TIRFM mode because of the accompanying improved signal to noise ratio. [3, 157] Due to the same effect TIRFM is restricted to studies of cell surface molecules as E-cadherin[164] or intracellular events taking place close to[165, 166] or at the cell membrane like vesicle fusion on the other hand. Synaptosomal-associated protein (SNAP) dynamics involved in exocytosis for instance where addressed by Wang *et al*. in an approach combining FRET and TIRF.[167] For further insights and recent instrumental advances as well as more application examples see the section dedicated to TIRFM in the comprehensive review by Stender *et al*.[3]

III.9 Imaging techniques just around the corner: SPRI, XRM, XAS, XRF

Despite of the impressing insights super-resolution fluorescence microscopy, electron microscopy and the other previously described advanced imaging techniques revealed up to now, each of them suffers from its own limitations. A step further or deeper always promises interesting new aspects – there is an unlimited demand for improvements and new developments. That is why two not that established single cell imaging techniques shall be shortly presented here.

A method promising to observe single cell reactions in real time and to be furthermore applicable in medical diagnosis is **surface plasmon resonance imaging** (**SPRI**). The surface plasmon resonance effect, the attenuation of light reflection at a phase border, results from resonance of an evanescent wave with a metal plasmonic field. A simple SPR setup might consist of a prism with a gold film on top. The evanescent wave develops if light passes from a medium with higher refractive index at an angle above a critical angle to a medium with lower refractive index. The plasmon is derived from electron gas movements in the metal layer. SPR happens at a specific angle of incoming light beam and phase border, the resonance angle. This resonance angle is highly sensitive to changes at the surface disturbing the plasmon which was exploited for long time in binding assays. [168] Capturing of cells based on surface molecule expression was described. [169] Recently the capability of SPR measurements to sense variations in single cell refractive index was reported and different SPRI devices were described [170, 171]. A review concerning the technique and its potential for allergy tests is available from Yanase *et al*. [168]

Insights into the presence, distribution and oxidation state of trace metals can be derived from **X-ray microscopy** (**XRM**), thus **X-ray absorption measurements** (**XAS**) and **X-ray fluorescence** (**XRF**). [172] Due to the availability of synchrotron radiation sources and improvements in X-ray optics, it is now possible to address subcellular compartments. XAS is based on the ejection of an electron from an atom shell at a certain energy amount which is reflected in the absorption spectrum. To enforce such an event sufficiently high energies are needed. The resulting electron hole is refilled by an outer shell electron which leads to element characteristic X-ray fluorescence (XRF) in addition. XAS can be operated in transmission or fluorescence mode. [173, 174] XAS images providing 50 nm lateral resolution and visualizing several organelles without the addition of contrasting chemicals have been published. [175] Further advances might be associated with the inset of nanoparticles [176] and lensless setups [177]. XRM convinces with respect to resolution, intrinsic contrast and tomographic capabilities. [178] However, X-rays will always be harmful for live. Thus, prolonged live cell studies stay unfavourable.

III.10 Multimodal imaging: correlating results from different approaches

Electron and X-ray microscopy allow high spatial resolution and excellent intrinsic contrast; however, sample cells do not survive these procedures. Raman spectroscopy and MSI datasets brim over from chemical information; but it is necessary to assign biological relevance to the detected changes in chemical composition. The diverse fluorescence based microscopy techniques deliver colourful images; however, they rely on artificial labelling and are blind for non-labelled subcellular morphologies. The question arises, if the image they depict is the truth?

Apart from the development of new and advanced methods in order to visualize finest structures and detect minimal molecule traces in front of overwhelming noise multimodal imaging approaches prosper. Multimodal imaging is the combination of at least two imaging techniques usually in a sequential manner. [2] That way, information derived from the cell is amplified at the one hand and integrated at the other one. Multimodal imaging faces its own complications. First of all, the desired techniques need to be compatible with respect to sample preparation requirements. Anyway, this will be often achieved by a reasonable technique flow. A fluorescence label will interfere with Raman measurements but a Raman measured sample is still suited for fluorescence staining. In general, the more invasive or even destroying method has to be applied as the final one. In contrast, the assignment of

corresponding datasets derived from different imaging approaches is inevitable. The development of automated registration algorithms is challenging due to varying magnifications, fields of view and underlying physics for the differing imaging techniques. [2, 179] Nevertheless, successful correlation of live cell confocal fluorescence imaging and SEM is described in a recent publication by Murphy *et al*. [179] Their multimodal approach alleviates feature identification in large data amount SEM stacks by registration of SEM data with fluorescing particles. Compatibility of Raman spectroscopic imaging and MSI was described by Li *et al*. [180] A recent review on correlative imaging focusing on the complementarities of the latter techniques is available by Masyuko *et al*. [2]

IV. Mapping the genome - tracing back the cell origin

The advent of DNA sequencing methods to identify the order of presence of the four different bases in DNA molecules opened the possibility to unfold the whole genome sequence of an organism. This facilitates the study of evolutionary relationships, genetic diseases and variations, and functional assignments of genes based on predicting their confirmation from their amino acid sequence. Genome sequencing of single cells only arose few years ago with the availability of less expensive and high throughput next generation sequencing techniques. Although mainly used to determine the diversity of non-culturable microorganisms in environmental samples [181], single cell genotyping can identify abnormal, mutated cells in a tissue or organism, which has a great potential for some diagnostic applications, especially to identify cancer cells or genetic defects in oocytes and sperms used for *in vitro* fertilization.

The main difference of single cell genome sequencing in comparison to sequencing a cell population is that the cells need to be isolated and that the genome needs to be amplified to obtain enough DNA for the sequencing process. This is usually done by multiple displacement amplification (MDA) that uses the DNA polymerase from phage Phi29 to copy the DNA with high fidelity[182] .

New technologies, such as combination of sequencing with microfluidics, allow sequencing at a subcellular scale, for example by separating two homologous copies of one chromosome from a cell in the metaphase, in order to determine different allele variations and meiotic recombinations[183]. For cancer diagnostics based on single cell genotyping, genome copy number quantification can reveal clonal subpopulations[184] , and sequencing of certain genes known to be often mutated in a special cancer type, can help to advance cancer diagnosis based on single cells[185] . In this regard exome sequencing is especially useful, as it allows studying more cells in a shorter time due to sequencing of only the protein-coding exon

regions of a gene. This makes it a convenient tool to study cancer development and the tumour biology in special cancer types in order to find common biomarkers and to identify frequently and less frequently occurring mutations[186, 187].

V An even deeper look at the molecular phenotype of a cell

Classically, the phenotype is known to comprise all the visible values of attributes of an individual in contrast or reflection of its genotype (see section III), where these attributes are coded. Visible characteristics of cells enable to separate them into groups, as epithelial or mesenchymal or, with more specificity, e.g. as glial or neuronal. More pronounced discrimination by presence or absence of certain molecules e.g. multi-drug resistance transporters, is also common and is especially important in pathology. However, the more molecular analysis techniques advance and the higher the throughput, the more attractive a separation according to the molecular phenotype becomes. The molecular phenotype, for the purpose of this review, is regarded as the sum of information concerning the presence of specific molecules (e.g. proteins, mRNA) as well as its overall chemical composition available for an individual cell.

Differential expression of genes gives rise to the diversity of cellular phenotypes. The presence or absence of their expression products - mRNA and proteins – is of special importance to characterize the molecular phenotype. With respect to mRNA studies, polymerase chain reaction (PCR), a technique well known from multi-cell analysis, has been adapted to be performed on single cell level. Advances in RNA sequencing and fluorescence in situ hybridization (FISH), however, make these approaches serious concurrence technologies. Improvements in spatial and temporal resolution in fluorescence detection systems have been achieved for RNA FISH and for the analyses on protein level, enabling new insights into cell cycle, signalling, behaviour and further more. Accordingly, proteomic and metabolomic information become more and more accessible even though, they are hard to assess on single cell level because their constituents cannot be amplified as easily as nucleic acids.[188]

A broader aspect, the overall chemical composition, is covered in the advancing field of biophotonics (section III). Spectroscopic data contain a spectral fingerprint of the cell, which also takes into account further cellular components as lipids in particular. In addition imaging of larger scenes with single cell recognition is possible.

V.1 Single cell gene expression analysis

The first step from genome to phenotype occurs at the level of transcription by copying the genetic information into a transportable messenger ribonucleic acid (mRNA) molecule. This mRNA then exits the nucleus and enters the translationary machinery of ribosomes in the cytoplasm in order to be used as a template for protein synthesis. This process is known as translation. Altogether, this gene expression process is pretty complex and the object of regulation at different levels and at several time points in various cell types underlining the importance to study gene expression on the single cell level.

There is a number of reviews concentrating on gene expression analysis at the single cell level, e.g., the ones by Tischler and Surani [189] or Stahlberg and Bengtsson [190] are recommended.

Multicellular organisms and tissues of higher animals are composed of many different cell types with the same genetic information but highly specialised in function. Thus, all these cells differ more or less in phenotype, making it necessary to study them on a single cell level to assess their function in the tissue and to understand the effects of stimuli from other cells (of same and different) types in the surrounding tissue. However, there are substantial cell-to-cell variations in gene expression even in cell populations of unicellular organisms with the same genotype.[191] These variations have been first observed in bacteria, but later also in eukaryotic cells. The variations can be described as the result of (1) extrinsic stochasticity, which is variation due to different activities of different regulatory molecules, and (2) intrinsic stochasticity, which is noise from gene expression itself due to random effects.[192] Stochasticity has been analysed in eukaryotic cells as well, and this revealed that gene expression frequencies and intensities can differ a lot between different eukaryotic genes. [191, 193, 194] Thus, single gene expression analysis is of utmost importance to identify gene regulatory networks by studying which genes are correlated in expression.[195] This cannot be achieved by whole cell population or tissue analysis because the signal derived from individual cell expression patterns will be averaged. RNAs expressed only in a few cells will be diluted so that their presence might not even be detected, thus making it difficult to elucidate co-regulation patterns.[196]

Single cell gene expression analysis is probably the easiest approach to obtain a comprehensive picture of the molecular phenotype of a single cell. This is due to the availability of very sophisticated and sensitive methods for the detection of RNA and the possibility to amplify the RNA amount in order to detect even low abundance RNA species.

In principal gene expression analysis can include a) the detection of certain mRNA species or the whole transcriptome in order to know which genes are transcribed in a certain cell type under certain conditions, b) the study of gene regulation to elucidate the connections of different regulatory proteins and gene sequences (such as transcription factors, microRNAs, epigenetics) in a cell and identify how they interact in order to influence gene expression, and c) the analysis of translation of mRNAs to proteins which reveals posttranscriptional regulation and numbers of proteins that are produced from one mRNA copy. This is important for functional validation of gene expression.

Already on mRNA level several methods can be used for single cell gene expression analysis that deliver quantitative and qualitative information about the presence of certain or all transcripts and that use different approaches (Table 2).

Reverse transcription quantitative real time PCR (RT-qPCR) and hybridization microarrays

Single cell RT-qPCR is a highly sensitive method that in principle allows for the detection of only one mRNA molecule. The method is quite suitable for the quantification of certain, selected mRNA species in many different single cells due to its high reproducibility and wide dynamic range.[190, 195] The cells are lysed, then, the released mRNA is transcribed into an identical DNA copy (cDNA) by reverse transcriptase, and finally, the cDNA is hybridized to a primer allowing its exponential amplification through DNA polymerase. The amplified DNA is detected in "real-time" by monitoring a fluorescence signal, either from a fluorescent intercalator or specific fluorescent tag. Since the resources and technical know-how for qPCR are widely established in many laboratories, RT-qPCR on single cells can be easily applied without high costs and efforts. For single cell analysis careful work is especially essential and any contamination in sample preparation must be avoided for an exact mRNA quantification due to the presence of only few mRNA molecules in one cell. Cell lysis should be efficient with maintaining the integrity of the RNA at the same time, and any RNA degradation in the lysate should be avoided by the use of efficient RNAse inhibitors. Further, reverse transcription should be efficient to make sure that all mRNAs get copied to cDNA, and it must be ensured that no primer-dimers are formed or unspecific amplificates are produced during to PCR, since they can obscure the exact quantification because of the low detection limit.[190, 195] Essential information for publication of qPCR results is given in the MIQE guidelines. [197]

An important drawback of single cell RT-qPCR in comparison to whole cell population RT-qPCR is the fact that due to the non-correlated and highly variable expression of different genes in different cells normalization to reference genes cannot be applied. This makes accurate quantification for comparison between different cell types difficult. A solution could be the addition of a known concentration of spike reference mRNA[190].

Further, with single cell RT-qPCR only 5 to 10 genes can be analysed in one cell without pre-amplification due to the relatively small sample size.[190] This might be overcome in future by using digital PCR. With that method single cDNA molecules can be detected. The sample from a single cell is diluted within miniaturized microfluidic devices to yield either one strand or no cDNA in one cavity where sensitive, automatic and reliable high-throughput PCR is carried out. [198] Another recent advancement of expression analysis aims at the transcriptome-wide analysis of the gene expression profiles by incorporating an universal PCR priming sequence via tagged priming and template switching.[199]

To date, microarray analysis offers a good way to study thousands of different mRNA species in one cell at the same time. This usually occurs through hybridization of the cDNA to specific oligonucleotide probes bound on a chip, and the resulting fluorescence-signal as read-out for quantification. Compared to PCR the disadvantages are possible cross-hybridizations and the low dynamic range, which can lead to falsification of the results.[196] For both PCR and hybridization microarray, the need for pre-designed oligonucleotides and a priori knowledge about the mRNA sequences makes it difficult to detect unknown RNA species as well as alternative splice variants that are only expressed under certain conditions.[196]

RNA Fluorescence In-Situ Hybridisation (FISH)

The principle of FISH is to hybridize a fluorescently-labelled oligonucleotide probe to nucleic acids, DNA or RNA. As for PCR a nucleotide sequence unique for the gene or gene product of interest compared to the rest of the genome or transcriptome is required for a successful FISH experiment. Specificity results from probe-target complementarity, thus, the definite base pairing between adenine and thymine on the one hand and guanine and cytosine/uracil on the other hand known from DNA amplification and its transcription into RNA is exploited. Possibilities to label probes directly or indirectly are diverse.[200] Originally FISH was developed to visualize certain DNA regions; oligonucleotides spanned several hundreds of basepairs (bp) for this purpose. In this field it rapidly evolved to paint whole chromosomes.[201]

Concerning mRNA analysis the key argument striking for FISH against PCR-based strategies is the delivery of spatial information. mRNA tracking from nascence to degradation would be, in principle, possible that way and it also makes single cell resolution an intrinsic feature.

RNA and DNA FISH can be performed next to each other, and also a combination with immunofluorescence labelling is possible. In a recent contribution Chatre and co-workers studied mitochondrial diversity in mammalian cells with respect to transcription and replication in that way and reported remarkable differences not only between individual cells but even between several mitochondria in one cell [202]. This gives an intriguing proof for the relevance of down-sizing experimental setups to single cell resolution.

The detection capacity of RNA FISH had been very limited until the end of the last century, due to fluorescence background noise. Singer and colleagues were the first who labelled short oligonucleotide samples (~ 50 bp) with up to five fluorophores to increase the number of fluorophores on a single mRNA target in order to detect it above the background.[203] However, the technique suffered from reduced binding specificity resulting from the high fluorophore load relative to the number of nucleobases and a difficult separation of completely fluorophore-conjugated oligonucleotides against only partly conjugated ones.[204] In 2008 Raj and colleagues described the possibility to shorten the oligonucleotide strand further, down to ~ 20 bp. This allowed the hybridization of even more probes to one target mRNA and therefore, the coupling of only one fluorophore to the 3' end of each probe. The reliability of probe binding was remarkably improved that way and diffraction limited single transcript visualization became reality. The simultaneous analysis of the expression of three different gene transcripts as well as the applicability in whole organisms was shown by these authors.[205] Sample barcoding, as suggested by Singer and co-workers, could further increase simultaneous detection capacities.[200, 203] Nowadays, single molecule RNA FISH serves to gain insights into reprogramming of fibroblasts to stem cells[206] or to assess stem cell markers in mouse intestine [207], just to name a two examples. Nevertheless, the shortness of recent oligonucleotide strands impairs hybridization selectivity. This is because of the increased probability of binding to highly similar or even identical nucleobase sequences in mRNAs belonging to different genes. The problem of high sequence similarity is circumventable to some extend by locked nucleic acids (LNA).[208] An approach to separate targets with single nucleotide difference was described by Larsson and colleagues[209]. The detection of single miRNA with a single single-labelled LNA probe has also been reported.[208] For more detailed information on single transcript FISH the review by Itzkovitz and van Oudenaarden is recommended.[204]

Fluorescent protein-based strategies

By using fluorescent fusion proteins, gene regulation mechanisms in living cells can indirectly be assessed over time. One example is the genetic engineering of fluorescent proteins that are fused to a repressor protein and a target protein, respectively. The regulation of the expression of the target gene by that repressor protein can then be investigated by following the fluorescent signal intensities.[210] Not only transcriptional regulation but also local translational regulation can be analyzed by using fluorescent protein engineering technology. Transfection of cells with mRNAs encoding fluorescent proteins can reveal the translational activity of these mRNAs in certain subcellular regions in polarized cell types. [196]

However, fluorescent fusion proteins tend to diffuse rapidly in the cytoplasm and can have a high stability, thus, making it difficult to study the temporal and spatial behaviour of gene expression in specific subcellular regions.[211, 212] Firefly luciferase offers a good alternative due to its short half-life making it very suitable to monitor fluctuations in gene expression. [212] Further, novel techniques were developed that allow direct fluorescent measurement of gene expression of single mRNA molecules.[211] Such a technique is the MS2 tagging system, a reporter system using transfection of a cell with two plasmids: one plasmid codes for the fluorescent-tagged capsid protein of the MS2 virus and the other plasmid contains the gene of interest with MS2 binding sites that allows binding of the MS2 protein to the RNA stem loop structure of the binding site.[211-213] Another example is the hybridization of mRNA using molecular beacons, single-stranded oligonucleotides tagged with a fluorophore and a quencher, that separate and thus give a fluorescent signal as soon as the oligonucleotide binds to the target RNA.[211, 214] Further, modified and new, improved fluorescent proteins with higher stability, less cytotoxicity, photoswitchability and near infrared excitation wavelength have been developed in the recent years allowing to monitor gene expression *in vivo*[212]. Together with the advancements in super-resolution microscopic techniques below the diffraction limit (see section III.8.4), these methods have great potential for high-resolution single-molecule studies in order to analyse the dynamics of gene regulation in single cells in the future.

RNA-sequencing

RNA sequencing (RNA-Seq) with the currently available next-generation sequencing approach is probably the method of choice to analyze the complete transcriptome of a single cell [196, 215]. To ensure that no sequences are missed the RNA must be pre-amplified either

through exponential amplification by PCR or through linear antisense amplification using primers with a binding site for T7 polymerase that ensures the maintenance of the relative mRNA amount. [215] Although the computational analysis and sequencing chemistries still need to be improved, the existence of specific algorithms for RNA-Seq data analysis already allows the extraction of usable information from the data.[196, 215] This and the additional functional validation of the transcriptome data are important for future applications such as the identification of potential therapeutic drug targets for the treatment of certain diseases [215, 216] or dissecting the transcriptome heterogeneity of mouse oocytes in order to understand the underlying developmental biology for advancements in stem cell research. [217] Furthermore, molecular labelling of each single mRNA in a sample with unique molecular identifies (UMIs) allows absolute and exact quantification of all mRNA copies by RNA sequencing because the quantitative information is reliably maintained during amplification by PCR.[218] This efficient combination of qualitative and quantitative mRNA determination holds the potential to powerfully advance transcriptome analysis in single cells.

Integration of gene expression analysis approaches

Currently, a decision has to be made between high-dimensional information on gene expression (array-based strategies or RNA-sequencing) where the cell has to be destroyed in advance and spatially resolved data on mRNA presence (RNA FISH or fluorescent protein-based approaches), that detect only few mRNA species at a time. Transcriptome wide and spatial information are combinable only if several cells are included, either in a bottom-up or a top-down way. Bottom-up means to start with knowledge on a confined number of transcripts and to build up a whole network, e.g., relying on interactions in a signalling cascade. While top-down refers to the in depth study of several transcripts whose relevance is concluded from transcriptome wide information derived at the beginning. For a confrontation of both strategies see Tischler and Surani [189].

Gene expression analysis on the single cell level was shown to advance studies that correlate small genetic variations with gene expression differences by showing differences not only between but even in the same individual [219] or that directly assess small nucleotide variations in RNA transcripts to analyze allele expression differences in single cells[220]. Ultimately, this may help to understand individual differences in organ functions and associated diseases.

V.2 Single cell proteomics

Although measurement of mRNA can already give a good hint on the protein expression diversity in a cell, it cannot tell about the quantity, location, protein-interactions and post-translational modifications of proteins [221]. This can be realized by the direct measurement of the protein composition in a cell. Whereas plenty of methods are available for efficient cell population protein analysis, single cell protein analysis is much more difficult, because of the small amount of proteins in a single cell that needs highly sensitive methods for detection. Especially the elucidation of the whole proteome is challenging due to its high complexity resulting from different organelle locations of the proteins (membrane-bound, nuclear, cytosolic proteins etc.), diverse post-translational modifications, protein translocations, and differing levels of activity [221].

Probably the most suitable method to analyse the whole proteome in this regard is mass spectrometry (MS), since it is label-free and can basically detect all proteins, post-translational modifications and peptides in one cell [221, 222]. In addition, MS-based proteomics allow the identification of endogenous protein interaction and modification during signalling [223]. For example, MALDI-MS (section III.7) and electrospray MS (see also section V.4) have been used for the analysis of certain proteins or peptides in single cells already. [224-226] Though, mass spectrometry has the disadvantage that it is not sensitive enough yet to allow detection of low abundance proteins. However, this can be improved by selective enrichment of cell subpopulation or cell fractionation, for example using microfluidics or FACS (see also section VIII.3/4). [221, 222]. Progress has been reported with respect to the successful proteome analysis of such pre-sorted subpopulations to answer specific questions (see Altelaar *et al*. [222] for a review of the studies).

Methods that employ separation of proteins are readily used on bulk protein samples; however they are difficult to apply on small protein samples from single cells. Microfluidic and capillary electrophoresis are able to overcome this problem [221], and successful attempts in this direction have been made, e.g. there are microfluidics available now to quantify low-abundance proteins. [227]

In contrast to proteomics, studying single proteins in single cells is more advanced: methods like flow cytometry or mass cytometry use specific antibodies and allow to study several proteins at one time (see also section VIII.3). Fluorescence-based arrays with antibodies bound to the surface can bind several proteins at the same time. This has been used to quantify intracellular signalling proteins in a cancer cell line [228] or to detect cytokines secreted from single cytotoxic T cells and other cells [229, 230] or mononuclear cells [231]. Further, a

combination with microfluidics to allow trapping, lysis and protein measurements in one system has been established and optimized recently as so called microfluidic antibody capture chips [232, 233].

In contrast to whole proteome studies, there are already numerous and multifaceted studies on the analysis of only a few, specific proteins in single cells. Those examples won't be discussed here, instead the reader is referred to the review of Wu and Singh.[221]

V.3 Single cell metabolomics

Single cell metabolomics addresses fundamental biological questions and is capable to observe metabolic phenomena in heterogeneous cell populations [234, 235]. The cell metabolome usually includes all intracellular and membrane-localized small molecules/metabolites with a molecular mass less than 1 kDa, e.g. lipids and carbohydrates. The metabolits can be exogenous, originating from outside the cell (as known as xenobiotics), or endogenous. Metabolites are involved in many intracellular functions and provide information of the physiological condition of the cell. Over the past few years metabolomic approaches developed rapidly and a number of useful databases, which store, manage and analyse the metabolomics data, occurred. The review by Go provides an overview of the recent progress in databases employed in metabolomics. [236] The most common techniques to measure the untargeted metabolome of tissue and other biological samples is the separation combined with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy [237]. Beside these detection techniques, the analyte extraction from the target cell becomes a crucial experimental step [73], because there is only a minute quantity of analytes in a single cell. Even the detection limit has been lowered from femtomoles to a low attomole range for single cell metabolomics [238]. Quantification is still problematic due to the need of conserving the original metabolome, which is often difficult because of the presence of enzymes in the sample and the fast metabolic turnover rates. [239] Microfluidics, gas and liquid chromatography and capillary electrophoresis as separation techniques in combination with detection methods like (laser induced) fluorescence or MS are the most promising techniques for single cell metabolome studies.

Mass spectrometry (MS) is an indispensable research tool in metabolite and peptide characterization. The capability to detect metabolites on a single cell level was partially described in section III.7 concerning mass spectrometry imaging (MSI) techniques. The workflow during MS measurements is almost the same: The analytes are transferred into the gas phase, ionized, separated and analysed by their mass-to-charge ratio and finally detected.

In addition to MSI methods, great affords in recent metabolomics studies based on MS techniques were made (reviewed by several authors[73, 234, 237]). Furthermore, Heinemann and Zenobi give an interesting overview of the current MS-based approaches for single cell metabolomics relating to their advantages and disadvantages [239].

One example for the increasing capability of single cell metabolomics via MS was shown by Nemes and co-workers by combining intracellular small volume samples with capillary electrophoresis (CE) and electrospray ionization (ESI) MS analysis. Thereby, over 300 distinct peaks were obtained in individual neurons. Furthermore, the identification of 36 intracellular metabolites and their quantitative analysis highlights the versatility of this technique[240]. For a detailed description of the CE-ESI-MS measurements of multiple metabolites including classical neurotransmitters (e.g. acetylcholine, histamine), energy carriers (e.g. adenosine) and osmolytes (e.g. betaines) among others in individual neurons from the sea slug (*Aplysia californica*) and rat (*Rattus norvegicus*) see the protocol from Nemes *et al*.[241] Intracellular sampling and high resolution ESI-MS detection of metabolites from single plant cells was done by Lorenzo Tejedor *et al*. [242] Oikawa demonstrated the utility of a large single cell model for an investigation of the metabolome and determined functional changes in the metabolite profiles of subcellular regions via CE-MS.[243] For more details concerning CE-MS see the review by Klepárník. [244]

In addition, flow cytometry (FC) with an extremely high throughput in single cell measurements in combination with MS detection offers the possibility to increase the number of independent measurement channels[237]. Mass cytometry, FC- Inductively coupled plasma (ICP)-MS, can use molecular probe labels containing rare earth elements[245]. Recognition of proteins with specific antibodies containing these elements, e.g. ytterbium 171 or neodymium, and their analysis via ICP-MS allows cellular antigen detection. Furthermore, a simultaneous quantitative analysis of more than 34 parameters, e.g. binding of 31 antibodies[246], cell viability, DNA content, and relative cell size at up to 1000 cells/s becomes possible[237]. Mass cytometric detection of metabolites as well as drugs including cases where the marker atom is incorporated into the analyte molecule itself, instead of in the affinity probe[247].

The combination of microfluidic devices and MS is seen as the method with the highest potential to deliver relevant data for systems biology. Thereby, single cell organisms are processed on the microfluidic chip for a quenching, lysis, and separation of the metabolites from the other cell components. Afterwards, a transfer to the MS devise takes place. Coupling of the microfluidic device to an ESI-MS is also possible. [239] The newest approach is the single cell elemental analysis via femtosecond laser ionization time-of-flight MS. [248]

Following mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy is the second most common technique to detect metabolites, however, so far mainly established for multicell analysis. Nevertheless, NMR can also be applied *in vivo* [72, 249]. The minimal sample preparation offers high-throughput studies. By its information-rich, reproducible and highly reliable character it is also capable to detect low-molecular-weight metabolites. For more details concerning metabolomics by NMR spectroscopy see the following reviews by Zhang *et al.* [250] and Gebregiworgis and Powers.[251]

However, due to its relatively low sensitivity NMR spectroscopy has reduced application possibilities on single cells. The studies by Grant *et al*.[252] and Lee *et al*.[253], which were already mentioned in the section III.6 concerning NMR imaging, are the most prominent investigations of NMR spectroscopy applied on single cells. Furthermore, achievements with new small-volume probe technologies, e.g. microcoils and microslot waveguide probes, enhance the detection limit and therefore potentially allow the characterization of cell-sized samples [73]. In conclusion, further efforts are needed to render NMR metabolomics applications of single cells.[234]

Furthermore, fluorometric metabolomics assays exist, which are generally based on the presence of fluorescent tags and a readout with an established technique, e.g. with fluorescence microscopy. The key advantages of fluorescence detection of intracellular metabolites include the high sensitivity, the capability to perform concentration dynamic studies, the nondestructive character and the high-throughput. Nevertheless, only a few metabolites can be analyzed directly in single cells by autofluorescence. In most of the cases a difficult labelling is required limiting the application capability. Especially fluorescent probes, which are expressed in living cells, can lead to an alteration of the native physiological status on the metabolome level of the cell - a further limitation of its applicability. Although nanosensor probes can be specific for different analytes, the number of simultaneously detectable components is limited as well. [234]

Electrochemical detection shows a high sensitivity making it capable for single cell analysis, even for quantitative studies. A label-free detection of intracellular and extracellular metabolites is possible. However, only electroactive species can be analyzed, which makes the electrochemical methods applicable only to targeted studies of metabolites in single cells. Nevertheless, monitoring of various physiological processes, e.g. release of catecholamines and oxygen, could be measured.[234]

Autoradiography and spectroscopic methods (Fourier transform infrared and Raman spectroscopy, see section III.4) are also applicable to analysis metabolites in single cells.

In summary, single cell metabolomics is still at the beginning of development and no in routine used method, as it is for macroscopic samples. Overcoming the listed limitations is the main challenge for highly sensitive, comprehensive and quantifiable single cell metabolomics assays. In most of the cases different techniques are available and can be also combined. One example is the detection of nitric oxide (NO), which is involved in a wide range of biological functions. There is an increasing interest in following nitric oxide synthases (NOS) activity directly by monitoring NO production, its function and metabolism [254]. Therefore, fluorescence imaging, CE–laser induced fluorescence and NO selective electrodes were successfully used for single cell NO production. Other methods, e.g. chemiluminescence, gas and liquid chromatography-MS were also applied, but are not as commonly employed for cellular and subcellular NO levels. In addition, the analytical techniques complement each other, e.g. electrochemical detection was combined with fluorescence imaging to study NO production in living systems with spatial and temporal specificity [254].

VI. Cell physiology and mechanics

Biophysical properties of cells can serve as label-free markers of the cells' physiological state. Understanding the changes in biophysical properties in single cells can contribute to understand human diseases. [255]

VI.1 Electrical properties

Electrical properties of a cell depend on the morphology of the cytoplasmic membrane, its lipid bilayer composition, thickness, and size as well as the ion concentration in the cell. Early models depicted the cells as a spherical body of cytoplasm confined by a thin dielectric membrane. [255]

Electrical properties of cells can serve as the basis for counting, trapping, focussing, separating and characterizing single cells. [255] Dielectric properties of a cell can be assessed in a non-invasive and label-free manner via alternating current (AC) electrokinetics and impedance measurements.[255, 256]

AC electrokinetic methods study the behaviour of the individual cells in an inhomogeneous electrical field. The cells experience a force and move (dielectrophoresis, DEP). If the phase of the electrical field is anisotropic the cell will also experience a torque and start rotating (electrorotation, ROT).[257] ROT is the only method which can determine intrinsic electrical properties of the cell such as specific membrane capacitance and cytoplasm conductivity and permittivity. [255] It was successfully applied to characterize leukocytes and human cancer

cells.[255] Drawbacks of this technique are the slow speed (30 min per single cell) and the limitation to low conductivity sucrose buffer solution. Physiological buffers cannot be used due to their high conductivity. [255]

The principles of impedance analysis of particles and the state-of-the-art in the field of microfluidic impedance flow cytometry can be found in a review by Sun and Morgan[258]. Impedance analysis can be carried out on flowing as well as on trapped cells. The Coulter counter was the first cytometer which could count and size individual cells based on their electrical properties (different resistance than the surrounding conducting fluid). It is still the dominating approach in the field[258] and was implemented into haematology analysers which are nowadays used in the clinics.[255] Miniaturized microfluidic Coulter counter entering the market now. However, those are unable to characterize the cell's electrical properties.

Microfluidic single cell impedance flow cytometry can reveal physiological information such as viability and membrane potential changes [259] as well as membrane capacitance and cytoplasm conductivity.[260] It has already been successfully used to obtain a differential count of leukocytes. [255, 261-263] However, up to now, it is difficult to correlate the observed electrical property changes to physiological changes in the cell. [255]

Microelectrical impedance spectroscopy (μ-EIS) probes the current response across a trapped cell.[255] Different techniques have been developed to trap the cells. Dynamic monitoring of electrical properties of the cell during growth or interaction with other substances is possible using either a microhole chip design or microelectrodes where single cells are directly grown on electrode holes or the electrodes themselves, respectively. [264-266] Different designs of such electrode traps are also possible. [267] However, the obtained parameters still depend on electrode size, cell trapping mechanism, cell volume and interactions between the cells.[255]

VI.2 Ion concentration, channel proteins and patch clamp

The effective ion activity in and around a cell plays an important role in determining the membrane potential and the rate of physiological interesting reactions. Therefore, the cells have the ability to actively modify the ion distribution by membrane channels and transporters. There are different methods to determine the ion concentration and follow the ion transport across the membrane involving radio-labelled tracers, ion-sensitive fluorescent indicator dyes, and ion-selective microelectrodes. [268] Ion-selective microelectrodes are glass capillaries with an ion-selective liquid membrane at the tip. These electrodes can be placed at the cell surface or inserted into larger cells. In combination with vibrating probe technologies, these electrodes can be even used to measure net ion fluxes. [268] (see also section VI.3) For

small single cells ion-selective microelectrodes are not the method of choice, but rather fluorescent dyes are used instead.

The gold standard method to study cellular ion channels is patch clamp which can provide highly accurate and rich information on ion channel activity and action potential via direct measurements. Whole cell patch clamp capacitance measurements can be used to study single exocytotic events in neuroendocrine cells, and was also combined with voltage clamp pulse stimulation and with stimulation by photorelease of caged calcium. [269] Further, ionic conductance in red blood cells was found to be mainly involved in pathophysiological scenarios.[270] Many modifications of the original patch clamp technique led to improved efficiency and previously unavailable data which is extensively used in cardiac cellular electrophysiology. [271] The application of electrophysiological methods to study transporters in native cellular membranes was recently reviewed by Grewer *et al*. [272] In combination with perturbation deep mechanistic information can be obtained.

In recent years, several attempts were carried out to improve throughput and make the technique available for characterizing drug – ion channel interactions. Innovative 'lab-on-a-chip' microtechnologies that modify design, fabrication, as well as enable microfluidic integration have been reviewed by Yobas. [273]

VI.3 Assessment of further physiological properties

For the sensing of biological relevant molecules such as O_2, NO, H_2O_2, ascorbate, glucose, dopamine, glutamate and ethanol microelectrode based approaches have been developed. [274] Further developments in this field do not only enable the determination of static concentrations, but also of the dynamic physiological flux. As this approach has been developed by several groups in parallel, it has many different names, such as vibrating probe, **self-referencing microelectrode**, microelectrode ion flux estimation and microelectrode flux estimation techniques.[19, 275, 276] Different variants include self-referencing amperometry where the analyte is either reduced or oxidized and self-referencing biosensors where electrochemically coupled enzymes are involved. [277]

While the electrode based techniques are limited to detect concentrations in the close vicinity of the cell, intracellular probes based on luminescence quenching are available for the **detection of oxygen** concentrations as well.[179] Very popular are luminescent metal (Pt, Pd, Ir, Ru) porphyrins whose luminescence lifetime and intensity can be quenched by molecular oxygen. [278] Also π-conjugated polymer nanoparticles have been proposed as fluorescent oxygen sensors. [279] These can be internalized into the cell by phagocytosis (especially investigated with macrophages), transport systems, such as microinjection, electroporation [280],

liposomal transfer, facilitated endocytosis [281], gene guns or with the help of special ligands that allow cell penetration [172, 282-284]. Application examples as well as a discussion of advantages and shortcomings of the different techniques have been given by Dmitriev and Papkovsky. [278] Multichannel biochips are under development for a parallelization of these techniques with the aim of achieving a higher sensitivity [169].

Calcium is an important mineral and **calcium ions (Ca^{2+})** play an important role as signalling "molecules". Therefore, elaborate techniques exists that can reveal calcium concentration and distribution inside living cells using confocal and two-photon fluorescence imaging (section III.8). Small molecule fluorophores that can chelate calcium ions or genetically encoded calcium indicators based on green fluorescent protein (GFP) are typically applied. [285] Calcium imaging is especially used to study neurons and neural activity. An extensive review is given by Grienberger and Konnerth. [286] Successfully calcium imaging in whole organisms was demonstrated within the commonly studied model organisms (see also see also section VII) *Danio rerio* (zebrafish) [287] and *Drosophila melanogaster* (fruit fly). [288]

Organic metal complexes have the potential to sense several other analytes as well. However, so far most of these studies were carried out under non-physiological conditions and cellular experiments have been limited to up-take experiments. [289, 290]

Maintaining the right pH in the cell and in the organelles is essential for the proper function of the cell. The **intracellular pH** can range from 4.7 in lysosomes to around 8 in mitochondria [291, 292]. Measurements of intracellular pH mostly utilize pH sensitive organic probes or fluorescent proteins that can be functionalized for specific cellular compartments. [292-294] Another approach uses SERS nanosensors [295].

Several other assays have been developed to detect and quantify analytes inside a single cell. The technology spectrum ranges from fluorescence to radiometric and enzymatic approaches employing different labelling and direct and indirect detection strategies. A detailed review of those techniques is beyond the scope of this review.

VI.4 Cell mass and water content

Refractometry, a technique relying on the refractive index, of a cell is done in a label-free way. In first approximation, the refractive index is dependent on the partial concentration of molecules in the cell. It has to be determined by a kind of titration of immersion liquid containing a known solid concentration against the cell compartment of interest. Under a phase contrast microscope the compartment will vanish as soon as the solid concentration in there equals that in the immersion liquid. Already in the 1950s refractive index measurement has been successfully introduced to cell biology for the study the cell mass and water

content.[296] Thus the refractive index serves to assess key physiological parameters. Refractometry has received certain attention during the last decade. Hilbert phase contrast microscopy which is used to acquire the refractive index map has been coupled to confocal reflectance microscopy which serves to extract information about the physical thickness of the specimen in order to improve the accuracy of refractive indices.[297] Furthermore, a tomographic three dimensional mode was introduced by Choi and co-workers that at the same time avoids keeping the cells in non-physiologic immersion substances.[298] The possibility to assess the chromosome mass by a derivative optical tomographic approach in a quantitative manner has been described recently.[299] Related to refractometry, Reed and colleagues implemented interference microscopy into this approach and studied the effect of drugs on a cancer cell line by quantifying the cell mass in a time dependent and high throughput manner.[300] A different setup, called spatial light interference microscope, was used by Mir and colleagues to measure the cell mass in relation to the cell cycle. Their development is applicable as an ad-on for a commercial microscope system allowing for combination of mass assessment and fluorescence acquisition.[301, 302]

Intrinsic physical properties of the cell, such as refractive index, composition, size, and deformability determine how a cell will react in an optical gradient field. Time-of-flight (TOF) optophoresis is used to probe the speed differences between different cells and by this to distinguish cell lines and drug-treated cells [142].

Suspended microchannel resonators were developed to determine the mass of a bacterium in water with sub-femtogram resolution [303]. Channel height limitations have been overcome so that now also eukaryotic cells can be weighted in such channels. Over 30 minutes the growth of individual cells (*Saccharomyces* sp. and mouse lympohblasts) could be followed by measuring the buoyant mass. Observing individual cells it was found that heavier cells grow faster than lighter cells. [304]

Furthermore, different mechanical resonator systems can be used to determine the mass of a cell placed on the surface of such a resonator [305]. The density of a single living cell can be measured by recording the mass of the cell of interest in two fluids with different density. [255] This was successfully applied to distinguish red blood cell associated disease such as malaria, sickle cell disease and thalassemia. [306]

VI.5 Mechanical properties

Cellular membranes, the cytoskeleton composition (both, structural proteins and cytoskeleton-associated proteins play a role) as well as size and density of the nucleus determine the

mechanical properties of the cell such as its deformability. Local measurement techniques, such as atomic force microscopy (AFM, see section III.2.1) [307], magnetic bead-based rheology or optical tweezers (see section VIII.1) [308, 309] and micropipette aspiration [310, 311] make it possible to probe mechanical properties of individual single cells also in liquids which resembles their natural environment. A summary of the functional range of different techniques to probe cellular mechanics is given by Loh *et al*. [312] A useful parameter to describe the elastic properties of cells is Young's modulus which, however, is dependent on various other factors. Therefore, same experimental conditions are crucial for comparability [313]. Probing intrinsic biophysical markers, such as elasticity does not require costly labels or extensive sample preparation.

Most research was done with cells for which deformability is of physiological relevance. Those are red blood cells (RBC), leukocytes and also cancer cells (potential circulating tumour cells) that have to squeeze through small blood vessels. Characterizing the cell's stiffness and deformability might give insights into different cell states and several human diseases, such as cancer, malaria, leukaemia, sickle cell disease, sepsis, hereditary spherocytosis, and diabetes. [255] Several studies proved alterations in mechanical properties such as cellular deformability to be useful to differentiate non-malignant and malignant cells. [313]

A simple way to squeeze cells is to force them through constriction channels which have a smaller diameter than the cells. High speed imaging can be used to follow transit time, elongation and recovery time. Electrical impedance measurements give transit time, impedance amplitude ratio and impedance phase increase. When only electrical readout is necessary the technique can be as fast as 100 cells per second. [255] Fluorescence measurements of labelled cells in the constriction channel can help to correlate mechanical deformability with already established cell surface markers. [255] One technical problem that might affect the measured values is that the friction between cell membrane and channel surface cannot be determined yet.

In order to study the deformability of soft and flexible cell types like erythrocytes, fluid shear stress in larger capillaries can be used. [255] Furthermore, optical stretchers were used to characterize the deformability of RBCs [314], human cancer cell lines and patient's oral squamous cells. [255] A dual beam trap acting as a cell stretcher was used to create controlled cellular deformation [315] and study the viscoelastic properties of the cell membrane in red blood cells. Malaria-infected RBCs were found to have increased rigidity due to the internalized parasite *Plasmodium falciparum*. [316]

When hydrodynamic stretching is applied to the cells, they are completely surrounded by liquid and do not have contact to the channels. High strains can be exerted on the cells which are easy to visualize. Gossett *et al*. successfully demonstrated the potential of this technique for the characterization of pleural fluid to determine leukocyte activation and cancer malignancy. [317] However, costly and bulky high speed cameras are needed that produce huge image data which require high computational effort for data analysis. Other potential applications of mechanical biomarkers in medicine are summarized by Di Carlo [318]. However, so far biomechanical markers hardly made it to clinical and biological applications. Further research needs to be done to better understand cell deformability changes as a function of environmental conditions and to improve the currently only poor correlation between cell deformability and the widely established biochemical markers. [255] Microfluidic developments will help to automate the analysis and enable high throughput. The different mechanical stimuli that can be implemented and probed in microfluidic systems to assess the cell deformability, including working mechanism, key observations and throughput have already been reviewed. [255, 319] They include, e.g., electroporative flow cytometry, DEP force as well as compressive forces applied through a thin membrane. The latter can be utilized to monitor cell viability and to induce mechanical lysis and in further modifications to reveal information about the viscoelastic properties of cells. [320]

The formation of bulges on the cellular membrane can be correlated to the cytoskeleton quantity inside the cell and used to distinguish breast cancer cells and normal cells [321].

VI.6 Binding and intracellular interactions down to a molecular level

In the previous sections a wide range of techniques has been already discussed that are able to investigate cell-substrate or intercellular interactions. Atomic force microscopy (AFM, section III.2.1) can measure inter- and intramolecular interaction forces with pico-Newton resolution. It could be successfully applied to measure interactions at the single-molecule level, e.g. to follow fibrinogen-platelet binding and fibrinogen-erythrocyte binding interactions which have relevance during cardiovascular disease. [322]

To monitor *in vivo* single molecule interactions, such as protein-protein interactions at the cell surface, single-molecule fluorescence resonance energy transfer (FRET) (section III.8.2) and fluorescence correlation spectroscopy (FCS) (section III.8.3) can be used [323, 324]. Further single-molecule mechanical assays are proposed to measure the in situ binding kinetics on the surface of live T cells. [325]

An immunohistochemical method for the detection of proteins and protein interaction is the in situ proximity ligation assay (PLA). Affinity reagents, such as antibodies, with an amplifiable DNA reporter molecule are used to visualize the protein of interest. [326-328] Steps towards automation in microfluidics have been taken recently. [329]

Surface plasmon resonance (SPR) (section III.7) combined with a special sensor and detector can be used to study the cell's response to stimuli, such as antigens, and follow the binding interactions. SPR imaging found already application for allergy screening by studying the response of human basophils to different antigens such as pollen antigens, mite antigens or sweat antigens [330] or by studying of rat basophilic leukaemia cell to immunoglobulin stimulation [171].

Another emerging field are targeted nanomaterials that are equipped with organelle-specific carriers and an effector molecule, e.g. designed nanoparticles for drug delivery. Important questions arising with such nanodeliverers are: Are there specific binding interactions? Are other parts of the cell also interacting with the target? Confocal fluorescence microscopy (section III.6.1) and high resolution TEM (section III.4) were utilized to visualize the interaction between drug-loaded nanoparticles and cancer cell nuclei. [331] Many further examples of nanoparticle-cell interaction exist.

A well-defined control can be gained over certain proteins such as light activated channels and enzymes by a technique called optogenetics. By genetic manipulation light-sensitive proteins are brought into the cell of interest which can then be switched on and off on a time-range of milliseconds. This allows the perturbation and subsequent detailed analysis of physiological processes. [332] Optogenetics is now widely adopted in neuroscience. [333]

Microfluidic developments (see section VIII.4) enable the measurement of many interaction forces of all kind, especially mechanical interactions can be characterized easily. Traction forces on microposts arrays can be used to estimate the strength of mechanical cell-substrate interactions as a function of morphology. [255] Furthermore, cellular response to external and internal forces can be followed in a time- and space resolved manner.[334, 335] Other microfluidic channels with fluid shear forces can be used to study adhesion forces of different cells, such as mammalian fibroblasts, activated and non-activated neutrophils and human breast cancer cells. [255]

A few other examples are given in other sections of this review (VI.2, VI.3, VIII) and can be found in the literature.

VII. The single cell in the multicellular organism

In a multicellular organism single cells have to act on an advanced level of cooperation. Often synchronization is of highest relevance, e. g., for productive heart muscle contraction and relaxation. Deviations may occur between *in vitro* and *in vivo* findings. An example is given by Yoo and co-workers who described differences concerning *in vitro* data and results derived from *Danio rerio* in *in vivo* studies regarding the position of the microtubule organizing complex in migrating neutrophils.[336] However, in agreement with *in vitro* data, recent publications bare intriguing variance in the molecular phenotype of cells belonging to the same tissue or sharing the same fate, respectively. Liu and colleagues described an approach based on **mRNA recognition** by fluorescence-labelled proteins to assess a cell position related gene expression heat map in *Cenorhabditis elegans* for 93 genes in 363 cells. They described differential gene expression between cells even belonging to the same syncytium in dependence of their respective lineages.[337] The single molecule mRNA-FISH approach described in section V.1 was as well shown to work in *Drosophila melanogaster* and *C. elegans*.[205] Variations in mRNA expression of a certain downstream effector (mec-3) for proper touch receptor neuron development in dependence of genetic background (alr-1 wild type and mutant) were studied in *C. elegans* larvae.[338]

Visualization and tracking of single cells in organisms becomes possible with new development in microscopic technique called **selective plane illumination microscopy (SPIM)** and its derivatives. For a review concerning SPIM see Weber and Huisken[339]. Due to special objective configurations which illuminate only a single object plane at once, SPIM avoids photodamage. In addition, time consuming scanning is evaded or at least reduced in dependence of the beam shape. SPIM application, though, requires the samples to be as transparent as possible to minimize photon scattering. It has already been used for detailed observation and visualization of neuron outgrowth during *C. elegans* development.[340] Furthermore, Krzic and co-workers tracked several cells during *D. melanogaster* development for five hours covering two cell division cycles.[341] Further improvements especially with respect to resolution, signal to noise ratio and artefact circumvention are highlighted in a recent contribution of Gao and colleagues describing their combinatorial approach of Bessel beam super-resolution structured illumination microscopy. They illustrate, e. g., *in vivo* karyotyping on the surface of a *D. melanogaster* embryo and mention the possibility to distinguish nuclei down to 20 µm in the sample.[342]

Single cell analysis in multi-cell surrounding mostly requires labelling of a cell type of interest. For a recent contribution reviewing several advanced labelling strategies see the one by Progatzky *et al*.[343].

Several **intravital microscopy** approaches were shown to be applicable for single cell observations as well. Especially **two-photon microscopy** gained relevance. It is based on the nearly simultaneous absorption of two photons by a single fluorophore. Both photons have to possess half of the energy that is necessary to raise an electron of the fluorophore to a higher energy level. Accordingly, photon wavelengths are doubled compared to single photon excitation of the same fluorophore. This has the advantage that the applied infrared wavelengths enable deep tissue penetration. The requirement of two photon absorption at once leads to intrinsic high confocality of two-photon fluorescence microscopy without the need for special optics.[344] In particular cells of the immune system have been the objective of single cell two-photon fluorescence microscopy.[345-347] As the basic review on two-photon laser scanning fluorescence microscopy the one by Denk *et al*.[344] is suggested for further reading. Concerning single cell studies by intravital microscopy a comprehensive review by Weigert *et al*.[284], containing a number of examples, is available and recommended.

A technique improving penetration depth of imaging is **photo-acoustic tomography (PAT)**. It is accordingly especially interesting for *in vivo* application. PAT exploits the transformation of an incoming electromagnetic wave to a density wave by an absorber, the opto-acoustic effect. A penetration depth of 5cm in tissue is enabled while a lateral resolution of less than 1mm is retained. For less penetration depth single cell observations are generally feasible if contrast is sufficient. [348] So far, PAT demonstrated its use for real single cell studies especially with respect to erythrocyte observations. By PAT based flowoxigraphy real-time oxygen release from erythrocytes in mouse brain was observed. [349] For a review on PAT and its subdivisions to complement optical imaging see Wang [348].

VIII Micromanipulation of single cells

Most of the micromanipulation techniques of single cells employ some sort of microfluidics because this enables an automated and user-friendly manipulation [350]. Quite often several manipulation tools are combined to comprehensively characterize and study single cells. Figure 3 schematises the most common applications which will be explained in more detail in the following paragraphs.

VIII.1 Trapping of single cells

There exist several methods to trap cells in solution and to keep them stable in space. Those methods apply optical forces using lasers, acoustic or ultrasound waves, dielectrophoretic forces or magnetic fields as well as hydrodynamic flows. [351]

Optical traps and tweezers

Lasers are able to exert forces in the range of femto Newtons to nano Newtons with a force resolution of 100 aN (sub-pN) and a time resolution in the range of μs. [142] These small forces are sufficient to manipulate inter- and intracellular processes and also to move microscopic cells. [142, 352, 353] In order to trap the cells, the surrounding medium should have a diffractive index less than that of the particle. [315] When applying lasers it is important to adjust the right parameters to avoid photodamage. Anaerobic conditions to avoid the formation of reactive oxygen species and the use of cold buffer to prevent heating effects were found to be beneficial. [142]

A single beam trap confines individual cells near the focus of the laser beam. Such traps can be easily combined with optical microscopy and various spectroscopic and optical imaging techniques such as fluorescence or Raman to further analyze the trapped cells. [315, 354] However, often traps use IR light which is not so suited for optical analysis and therefore, a second lasers is still needed.[142] Digital holographic microscopy was successfully applied to image optically trapped cells and to monitor their interaction with selectively moved particles with a temporal resolution of a few milliseconds. [355]

Dual fibre optical traps with two counter-propagating light beams cannot only trap larger cells than the single beam trap, but furthermore exert mechanical stress on the cells. Such traps can be easily implemented in microfluidic with orthogonal viewing. [315] This enables the study of mechanical properties of cells (section VI.2) or certain properties of cells under mechanic stress, such as, e.g., calcium signalling of human embryonic kidney (HEK) cells. [356]

Multiple optical tweezers can be combined to control the spatial position in 3D of hundreds of cells at a time to improve throughput. Methods to create multiple traps have been reviewed by Ramser and Hanstorp. [142]

Holographic optical tweezers can be used to create specific cell arrangements to study the influence of a certain position or interaction, [142] and to orient the cell or some of its organelles. It was, for example, possible to arrange the nuclei of different individual cells all in the same plane or move free-lying vesicles in the cytosol of NG-108 cells [357]. Movements of beads functionalized with secretory molecules towards neural cells can be even used to

stimulate the neuronal growth. [358] In addition, single molecules (e.g. kinesin molecule) inside living cells can be monitored with optical tweezers. [359]

Not only lasers can be used to generate optical fields, but also arrays of gold micro-pads. When light couples to the surface plasmons, cells can be trapped with those **surface plasmon tweezers**. [360-363]

Acoustic trapping

Ultrasonic waves were also successfully used to position cells in the centre of microwells and further investigate them there. [364-366]

Other, non-optical trapping methods make use of the hydrodynamic flow (hydrodynamic trapping) which can be implemented in three-dimensional microfluidic system, or electric fields (**Dielectrophoretic trapping, DEP).** For the latter one, the electric field should not be too high in order to keep survival rates high. **Magnetic tweezers** employing magnetic nanoparticles can be used to manipulate individual molecules inside a single cell. [367]

VIII.2 Invasive manipulations

Optical scalpels: Pulsed UV-laser or NIR laser light can be focused with high precision on certain organelles or even molecules. If the energy of the laser is high enough it can act as an optical scalpel which can knock out molecules or irreversible impair organelles.[142] This was applied to damage DNA to study subsequently the DNA repair mechanisms, to photoporate cell membranes for transfection experiments (References in [142]) and to disrupt individual mitochondria in living HeLa cells [368]. Small holes in membranes induced by UV illumination can be used to study the diffusion of small fluorescent molecules and probe compartmentalization. Furthermore, targeted lysis of very small cell regions is possible with a high precision in space and time. [142]

Electroporation can be used to introduce foreign molecules (DNA, proteins) into cells by temporarily disrupting the cell membrane by a voltage shock [255]

Single cell lysis can be achieved by different stimuli: laser pulses, electrical pulses and other electrical pertubations, sonication, detergent/surfactant, and chemicals. The characteristics of those techniques regarding time, platform and denaturating influence on cellular structures and features have been reviewed by Brown and Audet.[369]

VIII.3. Separation and sorting

Optical traps can be arranged to generate optical landscapes in which cells experience different optical forces according to their shape, size and refractive index. These forces can be used for passive, light-induced separation of cells. [142]. This technique worked fine to separate red and white blood cells. For very similar cells the low sorting efficiency can be improved by binding dielectric microspheres to the cells of interest. [142]

Electrophoresis is an electrokinetic phenomenon exploiting the movement of dispersed particles in an electric field. This technique has been first employed to separate small molecules, but is now also used for single cell experiments and to profile the many different analytes in a single cell. [370] Parallel detection of more than one analyte is possible in a large dynamic detection range, opening the way to functional metabolomics studies. [371]
With capillary electrophoresis (CE) only low sample volumes are required and a fast and efficient separation of the components within single cells can be achieved.[372] The technique is portable and very versatile and can be coupled with microfluidics. Common analysis methods used in combination with electrophoresis are fluorescence (laser-induced (native) fluorescence: CE-LINF and CE-LIF, respectively), electrochemistry (EC, amperometry, voltammetry, and conductivity), and mass spectrometric detection; less common methods are based on radionuclide and nuclear magnetic resonance.[372]

Flow cytometry is a high throughput technique that uses cellular characteristics such as morphology or fluorescence (labels) to sort, count and purify cells and to determine the cellular phenotype. Detection is mostly done optically or electronically. Furthermore, flow cytometry enables quantitative analysis of protein expression, protein epitopes, protein phosphorylation state, nucleic acids, and ion concentrations in single cells. [373] However, only a snapshot in time is acquired, continuous monitoring of individual cells over time is not possible. Nevertheless, the high throughput of the technique enables to investigate the heterogeneity among a cell population. [374] Flow cytometry is already routinely used for diagnostics in haematology and immunology, as well as in cell-based basic research. Cell function and cell properties, such as abundance of special proteins [375], occurrence of reactive oxygen species, viability state and others can be determined.

Fluorescence-activated cell sorting (FACS)

The acronym FACS is a trademark and was introduced by the company Becton Dickinson, but is nowadays often used in a generic sense. [376] FACS uses the light scattering and fluorescence properties of the cells to sort them into subpopulations relying on user defined criteria[374]. Polychromatic flow cytometry uses more than 6 colours at the same time. Nowadays, with the help of fluorescence labels 18 different proteins per cell can be quantified at a rate of >10 000 cells/s. [377] This can yield deep insights into immune cell subpopulations and immune cell function.

Magnetic affinity cell sorting (MACS)

As FACS also the acronym MACS is a trademark (by the company Miltenyi). Instead of fluorescence labels magnetic beads are selectively attached to a specific antigen or cell surface marker of the selected cell subpopulation. The cell separation and purification can be carried out in positive selection mode (selected cells carry the bead) or negative selection mode (selected cells do not carry the bead).

Single cell mass cytometry

Mass cytometry is a relatively new version of flow cytometry that combines it with mass spectrometry. Theoretically, it is possible to differentiate 70 – 100 parameters in a quantitative and specific manner over a high dynamic range with high throughput (1000 cells per second).[245, 377] Instead of fluorescence labels, purified, stable (non-radioactive) isotopes of non-biological, rare earth metals (typically lanthanides) are tagged to antibodies and/or DNA intercalators and used as reporters. For analysis, the cellular material of a single cell is nebulized and analyzed with a time-of-flight mass spectrometer. Unlike conventional flow cytometry methods, single cell mass cytometry is not suitable for work with living cells and it is impossible to recover live cells back after the mass cytometry experiment. Further comparison of fluorescence based and mass cytometry can be found in the review by Bendall *et al*. [377] Applications lie in the field of immunology, stem cell research and haematology.[246]

VIII.4 From microfluidics to lab-on-a-chip

Microfluidic systems usually consist of a structure of channels (typically in the micrometer range: 10-100 μm) that can be designed individually for each experiment and equipped with functional structures (capturing elements, antibodies, electrodes, etc.). Functional assays or the combination with other experimental cell analysis techniques are thus permitted. [142]

Common names for these integrated microfluidic concepts are lab-on-a-chip systems or micro-total analysis systems (μ-TAS).

Microfluidic systems have many advantages for single cells studies and therefore, have undergone a fast technical development during the last years. Only small sample volumes are necessary saving expensive reagents; increased (multi-step) integration and automation capabilities make the assays user friendly; fast response and increased sensitivity bring reliability and statistical information and environmental parameters (pH, salt concentrations, drugs, temperature and others) can be controlled precisely. [255, 373, 378] Ultimately, microfluidic systems might mimic certain *in vivo* situations in an *in vitro* setting. It is already possible to follow dynamic events and cell-cell interaction.

Many of the single cell analysis techniques described in this review have been transferred into microfluidics where sub-cellular information can be analysed by various high-contents analysis methods under defined cellular environments and stimuli. [379, 380] Single cell gene expression measurements including measurements of expression dynamics, high-throughput single cell RT-qPCR, transcript multiplexing, single cell whole genome analysis, protein analysis, signalling response and growth dynamics analysis as well as biophysical measurements could be already successfully incorporated into microfluidics. [255, 373, 381] Intracellular protein expression and the release of cytokines and effector molecules can be visualized by combining microfluidics with single molecule imaging, fluorescence imaging as well as with miniature antibody arrays. [228, 373, 382] This was shown first for bacteria but could be transferred to eukaryotic cells as well. Protein abundances were found to vary from 0.1 to 10^4 molecules per cells. Furthermore, mRNA and protein abundances seemed to be rather uncorrelated indicating a rapid degradation of mRNA. The real-time monitoring of the release of signal molecules such as NO, insulin, Ca^{2+}, neurotransmitters or histamine from single living cells in a microfluidic set-up has been reviewed. [383] Observations of single cells in microfluidic systems can further help to set up detailed kinetic models for cellular reactions and metabolics as was shown for the dynamics of glycolytic oscillations in single yeast cells. [384]

In a special version of lab-on-a-chip technologies, called droplet microfluidic, single cells are encapsulated in individual liquid containers which can act as carrier as well as microreactors. [385] Within those picoliter-sized droplets that can be generated within a few milliseconds with high monodispersity, otherwise undetectable signals of single cells, such as rare secretions, become concentrated to measurable levels. [385, 386]

Despite all the progress, in most examples, sample preparation for microfluidic analysis is still carried out off-chip on the benchtop, requiring instruments such as centrifuges to separate cells from surrounding body liquids etc. [387] This extra working step requiring extra man power and causing irreproducibility in the results so far prevented the entry of microfluidics into routine diagnostics. First attempts to integrate all sample handling steps could be shown for RT-qPCR on microfluidic chips achieving a throughput of 300 cells/run [388] and for on-chip cellomics.[389]

Applications of lab-on-a-chip systems range from basic research in proteomics and immunocytometry to haematology, human haplotyping, drug discovery and development, biosensor applications as well as stem cell and cancer research. [373, 390] Clinical impact could be generated from predictive gene expression and intracellular signalling protein signatures. Furthermore, cellular heterogeneity could help to predict disease progression, optimal treatment strategy as well as patient survival and outcome for cancer patients. [373, 382] Furthermore, the organization of cells in complex arrays for the creation of synthetic tissue seems possible. [142]

Microelectromechanical systems (MEMS) have been developed to study the mechanobiology of living cells in microengineered platforms under close to *in vivo* conditions. [311, 391] This shall help to elucidate underlying sensing mechanisms and force transduction of cells under various mechanical stimuli which can occur in a human body, such as muscle action, heartbeat, lung action or shear stress in blood vessels, but also during cancer cell dissemination. [311] Ultimately, this will lead to a more complete understanding of how cells function. Several strategies for cell-biomaterial interactions have been developed to assure biocompatibility of the MEMS material. [392, 393] Advancements in MEMS technology allow the fabrication of cell size matching devices such as microscale electrodes and arrays for precise manipulation with spatially and temporally variable stimuli and quantitative evaluation of cellular response. [311, 391, 394]

IX. Classification or what characterizes a cell type

The amount of different data collectable from an individual cell is incredible. If the techniques available for *in vitro* studies are combined in a reasonable sequence, values on its mass, chemical composition and expression level of hundreds of genes are detectable. Making concessions at the number of gene products accessible time resolved studies on the impact of manipulations are possible. Thus the detailed molecular phenotype for each cell of interest is

available in principle. But what is the excess value? What one can conclude from high end statistical analysis? As discussed before, there is no cell like the other, there is always biological variation. This biological variation is observed for all measured parameters, like size, mass, gene expression patters with different amount of transcripts and synthesized proteins, as well as different levels of metabolites or the slightly different reaction on certain stimuli. This raises the questions, how big has the difference between two cells to be in order to define a new cell type? How big is the acceptable variation in morphology and phenotype within one cell type? What is just stochasticity, or noise, in gene expression due to different transcription rates, regulatory dynamics and genetic factors?

For very different cells the belonging to different cell types is widely accepted, e.g., for healthy cells, that are integrated in the optimized function of the organism and tumour cells, that ill-behave and perturb normal function. Several analytical methods are currently researched and established to differentiate and sort such healthy and malignant cells based on relative drastic changes in metabolism and gene expression upon the acquirement of malignancy. Polymerase chain reaction (PCR, section V.1) [395], hybridization microarrays (section V.1) [396], hyperspectral imaging (section III.8) [159] Raman spectroscopy (section III.4) [354] as well as refractometry (section VI.4) [397] were effectively applied for discrimination, to name just a few examples.

The situation of differentiating cells into different cell types gets more complex if we have a closer look at, e.g., lymphocytes and their various subpopulations. Immunologists use different surface markers to distinguish a huge variety of different cell types based on different surface marker expression patterns. Variations and a high heterogeneity are also observed if the interaction of e.g. isolated and purified natural killer (NK) cells and target cells is investigated. While some NK cells exhibit high cytotoxic activity and killing efficiency others show less or even no killing activity. Based on the interaction behaviour the NK cells could be divided into further subgroups. [398] Up to now it is not clear what determines those activity differences on a phenotypic level.

In order to elucidate the critical difference between two different cells and identify what is only a less important detail and could be assigned to biological variations, statistical data analysis methods can be applied to search for inherent structure in the data set. Unsupervised statistical algorithms, such as principal component analysis have been successfully applied to cluster results of single cell analysis methods, such as e.g. vibrational spectra and gene expression profiles. Looking at primary mouse astrocytes unsupervised analysis of gene expression profiles revealed two distinct astrocyte subpopulations. [399] In the same study, gene

correlation algorithms were used to identify differences in the activity of important transcriptional pathways.

During lineage pursuance classification is also of interest, and was done in whole organism context in *C. elegans* based on gene expression analysis by Liu and colleagues [337] Again, the same intriguing question of single cell classification arises: How huge does the difference in molecular phenotype between two individual cells have to be, to assign them to be of unequal cell type? From the opposite perspective: Up to which degree of difference in molecular phenotype it is just a sign of variance of one cell type? Probably the answers to these questions won't even be the same for each cell type. There may be cells of high specialization that are highly similar one to the other, inner ear hair cells might serve as an example. On the other hand, e.g., hepatic stellate cells are known for a dramatic phenotype transition when they switch from quiescence to activation which is reflected in gene expression[400] and vibrational spectroscopy data[41].

So far, a convincing concept how to handle the cell type term in the classification context does not exist. It is expected, that with a better understanding of single cells, their organization and function, we may better define new markers and characteristics that allow classification of cell types and a sub-types.

X. Single cell analysis for diagnostic use

Single cell research shall not only bring new insights into the mechanisms of life, but there are also some hopes that the results could be utilized in medicine. The advantage of basing the diagnostics on single cell analysis is that only minimal sample (e.g., very tiny biopsy) is required from the patient and point-of-care devices for personalized medicine might become feasible. While some of the presented single cell analysis techniques are already quite advanced and are in or close to medical applications, such as single cell gene expression analysis (see section V.1), other techniques still struggle for medical relevance, such as the biomechanical markers (see section VI.2). Microfluidic developments, advances in imaging industry and hardware based image processing are expected to help with automated analysis and higher throughput providing the necessary statistical accuracy for an ultimate clinical diagnostic application. High expectations are also raised to integrated analysis techniques where two or more of the presented techniques are combined to provide an even deeper insight into the single cell characteristics.

Key fields of application that can be seen from today are oncology, especially in the field of tumour diagnostics and circulating tumour cells, haematology, regenerative medicine (pre-

implantation diagnostics and embryonic stem cell research), drug development and immunology. A few examples from those fields will be highlighted in the following.

Circulating tumour cells (CTC) can be found in the peripheral blood of cancer patients with metastasising primary tumour. It was shown that the number of CTC can give valuable insights into disease severity; change in number can report the success of therapy, while the identity of the CTC could lead to the primary tumour. Different detection methods for CTC from blood have been developed within the last years. [401] Single cell polymerase chain reaction (PCR) (section V.1) can distinguish and determine different tumour cells such as CTC, but also cancer stem cells and thus, help to find the best therapy for the patient. One of the problems with CTC is that they are only present in low number in a heterogeneous sample (the patient's blood) together with many other non-tumour cells. Therefore, high throughput techniques are needed. Statistically meaningful data require the analysis of large cell numbers. Lab-on-a-chip technology made huge advances in the last years and now allows efficient capture, separation, enrichment, detection and count of different CTC with high throughput rates and different subsequent analysis methods. [402-405]

Not only for freely circulating tumour cells which already exist in the single cell state, but also for solid tumours and **tumour research,** single cell analysis proofed to be beneficial and to hold clinical potential. For tumour diagnosis only a very small biopsy, which has only minimal impact on the patient, has to be taken if single cell analysis methods are employed [195]. Insights into tumour heterogeneity resulting from genetically and phenotypically different tumour cells can be gained by means of single cell gene expression analysis.[195] Promising examples are the use of single cell analysis for the discrimination of colon cancer tissue from healthy epithelia by RT-qPCR [406], and the RNA-Seq transcriptome analysis of circulating tumour cells from solid melanoma. The latter study analyzed CTC heterogeneity and aimed to identify potential biomarkers [407]. Different approaches for tumour analysis on the single cell level have been reviewed by Bendall and Nolan [408]

In **regenerative medicine,** the analysis of pluripotent stem cells that are used for differentiation into certain cell types for treatment of diseases is of utmost importance. It is necessary to check their homogenous differentiation into the desired phenotype.[195] For example, concerning the heterogeneity among different neuronal progenitor cell types the transcriptome can give significant information about their cell fate and development. This is fundamental for the development of future stem cell based therapies. [409-411] Similarly, assessing the transcriptomal heterogeneity in single cells can be used to track and understand the regulation of the differentiation process of hematopoetic stem cells.[412,413] Single cell PCR

is already widely used for pre-implantation genetic diagnosis to identify embryos with genetic diseases or abnormal chromosome numbers.[195] Not only transcriptome based analyses find application, but also the real-time tracking and fluorescent ratio imaging of sperm motility and energetics are carried out in automated systems in human fertility clinics and animal breeding farms [414] Manipulation of single cells with optical tweezers is conceivable for *in vitro* fertilization, stem cell research, and single cell transfection. [142, 353]

Single cell analysis can also have an impact on **drug discovery.** Integrative 'systems pharmacology' strategies use the complex response (e.g. gene expression, metabolic states or cellular phenotype) of a single cell to understand the action of a drug. Bioinformatics methods are able to extract information about the drug's targets, mechanism of action, metabolism and toxicity from these multi-parametric readouts in data-driven computational approaches. [415] Single cell transcriptome analysis provides detailed new insights into intercellular variability of the RNA profiles and thus, helps to identify previously unrecognized drug targets. [215] For example, RNA-Seq transcriptome analysis of neuronal cells, which are highly specialized and polarized cells [196], is of great benefit for the field of neuropharmacology, because it can be used to identify new receptors and channels as potential drug targets in neurological diseases. [216]

Also mechanical characteristics of a cell (see section VI.2), such as the simple deformability which can be probed label-free, i.e., without costly antibodies, have a high potential for drug discovery and personalized medicine. Measure of cytoskeletal integrity could enable screenings for drugs interacting with actin or tubulin of the cytoskeleton, as well as the detection of resistances against those drugs in biopsied samples.[317]

Applications of single cell analysis methods in the field of **immunology** comprise, amongst others, gene profiling of induced T or B cell subsets of the adaptive immune response for the discrimination of special vaccination-induced $CD8^+$ T cell subpopulations.[416] Furthermore, several flow cytometry based assays have been developed to determine the immune status of transplantation patients. [417] Research that could translate into a better understanding of the immune response and therefore, into the improvement of therapy designs, includes the study of the immune activity of single natural killer (NK) cells against target cells (virus-infected or cancer cells). Significant differences in the number of contacts between NK cells and target cells as well as in the killing efficiency could be revealed. However, those results are still waiting for an ultimate answer. [398, 418]

XI. Conclusion

Studying single cells offers valuable insights into spatio-temporal dynamics of biological processes and interactions, even down to the molecular level. An amazing variety of different techniques and methods already exists to visualize morphology, phenotypic characteristics and gain insights into the genome of a single cell. Despite all the advances, many interesting questions remain unanswered, such as detailed signal transduction or the exact role and timely arrangement of all the involved molecules, clusters and organelles in different interaction mechanisms. In order to answer those questions further technological development and advanced data evaluation strategies are required. The combination of different single cell analysis methods is evolving which can provide global, integrated and multi-parameter information from an individual single cell which is necessary to understand the complex organization of life. Microfluidic systems already bring together different sampling techniques to collect biophysical and biochemical information. Further promising combinations could involve super-resolution microscopy, and novel biochemical and single-molecule kinetic measurements with an improved temporal resolution. In order to efficiently utilize all the different techniques a vivid interdisciplinary exchange among specialized scientists is required to enable the physicists and engineers to design valuable tools and devices which are able to answer the exciting biological questions of life scientists. Extensive computational effort is also required to extract the important correlation and information from the data flood and the noisy and complex signals that show such high cell-to-cell variations. System biologists will contribute valuable models that describe the complex interaction networks observed in the experiments and help to understand which parameters determine the reaction and in the end the fate of the cell.

All future technological improvements have to bear in mind that the amount of sample from a single cell is limited, therefore, asking for sophisticated detection performance. In addition, since intrinsic variability between cells is large, high-throughput techniques are a pre-requisite for reliable conclusions to be made from the analysis of large numbers of individual single cells. Finally, the realization of single cell studies in the natural cell niche, such as within tissues, is desirable.

Ultimately, the results from single cell research and analysis have great future potential for application in personalized medicine with new point-of-care devices that make use of novel prognostic and predictive biomarkers. This might help, for example, to identify key mutations in cancer genes or physiological parameters of certain disease states.

Acknowledgements

Financial support by the BMBF (FKZ 01EO1002, Integrated Research and Treatment Center "Center for Sepsis Control and Care") and the DFG via the research group FOR 1738 "Heme and heme degradation products" is highly acknowledged. Furthermore, we thank Rainer Heintzmann and Marie Walde for discussion on high resolution fluorescence microscopy and figure 2.

Figure captions

Figure 1: Scheme of a single cell with its components (drawn not to scale) and an overview of the techniques that can be used to investigate and characterize the cell.

Figure 2: Illustration of the spatial resolution of the different microscopy techniques using the example of the podosome ring of actin associated proteins in THP-1 cells (macrophages). The membrane-cytoskeletal protein vinculin was labelled with Alexa488 (A-C, E) or ATTO-647 (D) for visualization. All scale bars are 2 µm. (Figure adapted from Walde *et al.* [419] and Cox *et al* [158]).

A: Widefield image of a macrophage. The cylindrical actin-rich podosomes (0.5-2µm in diameter) can be seen as bright circles against a blur background. (section III.8)

B: Confocal image, as the podosomes are very thin structures with only minimal depth profile (they form a flat sample with all the podosomes close to the cover slide surface) the sectioning difference between widefield (A) and confocal (B) is relatively small. (section III.8.1)

C: SIM image. (section III.8.4)

D: STED image recorded on a commercial instrument. Even better resolution can be achieved with home-build STED systems. (section III.8.4)

E: 3B image. No background fluorescence interferes with the signal from the podosomes. (section III.8.4)

Figure 3: Overview of single cell microfluidics applications.

Table caption

Table 1: Imaging methods grouped according to required contrast.

Table 2: Performance parameters of gene expression analysis methods.

Table 1: Imaging methods grouped according to required contrast

Label-free methods (internal contrast of the sample)	**Contrast-enhanced methods (specific label is applied)**
Optical microscopy (without stains)	Optical microscopy (with dyes to stain special organelles)
Scanning probe microscopy	Fluorescence microscopy
Electron microscopy	Nuclear magnetic resonance imaging with paramagnetic metal particles
X-ray microscopy	
Linear and non-linear vibrational spectroscopy	
Mass spectrometry	

Table 2: Performance parameters of gene expression analysis methods.

technique	excessiblity of different transcripts	information on spatial occurence	single transcript sensitivity	speciality
RT-qPCR	about 10	no	yes	equippement from large scale RT-qPCR applicable
RNA-FISH	less than 10	yes	yes	super resolution microscopy necessary for advanced studies
hybridization microarray	several thousands (pre-amplifiaction assumed)	no	no	transcripts of high sequence similarity hardly discriminable
protein based strategies	less than 10	yes	yes	transfection required
sequencing	whole transcriptome (pre-amplifiaction assumed)	no	no	no pre-definition of relevant transcripts required

References

1. C. Petibois, *Anal. Bioanal. Chem.*, 2010, **397**, 2051-2065.
2. R. Masyuko, E. J. Lanni, J. V. Sweedler and P. W. Bohn, *The Analyst*, 2013, **138**, 1924-1939.
3. A. S. Stender, K. Marchuk, C. Liu, S. Sander, M. W. Meyer, E. A. Smith, B. Neupane, G. F. Wang, J. J. Li, J. X. Cheng, B. Huang and N. Fang, *Chem. Rev.*, 2013, **113**, 2469-2527.
4. M. Mir, B. Bhaduri, R. Wang, R. Y. Zhu and G. Popescu, in *Progress in Optics, Vol 57*, Elsevier Science Bv, Amsterdam, Editon edn., 2012, vol. 57, pp. 133-217.
5. J. L. S. Milne, M. J. Borgnia, A. Bartesaghi, E. E. H. Tran, L. A. Earl, D. M. Schauder, J. Lengyel, J. Pierson, A. Patwardhan and S. Subramaniam, *Febs J.*, 2013, **280**, 28-45.
6. A. A. Sousa and R. D. Leapman, *Ultramicroscopy*, 2012, **123**, 38-49.
7. L. F. Kourkoutis, J. M. Plitzko and W. Baumeister, in *Annual Review of Materials Research, Vol 42*, ed. D. R. Clarke, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 42, pp. 33-58.
8. T. Yahav, T. Maimon, E. Grossman, I. Dahan and O. Medalia, *Current opinion in structural biology*, 2011, **21**, 670-677.
9. J. Pierson, M. Vos, J. R. McIntosh and P. J. Peters, *Journal of electron microscopy*, 2011, **60 Suppl 1**, S93-100.
10. I. Hurbain and M. Sachse, *Biology of the cell / under the auspices of the European Cell Biology Organization*, 2011, **103**, 405-420.
11. E. V. Orlova and H. R. Saibil, *Chem Rev*, 2011, **111**, 7710-7748.
12. A. Hoenger and C. Bouchet-Marquis, *Adv Protein Chem Struct Biol*, 2011, **82**, 67-90.
13. A. Hoenger and J. R. McIntosh, *Curr Opin Cell Biol*, 2009, **21**, 89-96.
14. C. Batchelor-McAuley, E. J. F. Dickinson, N. V. Rees, K. E. Toghill and R. G. Compton, *Anal. Chem.*, 2012, **84**, 669-684.
15. Y. F. Dufrene and A. E. Pelling, *Nanoscale*, 2013, **5**, 4094-4104.
16. X. L. Shi, X. J. Zhang, T. Xia and X. H. Fang, *Nanomedicine*, 2012, **7**, 1625-1637.
17. T. Ando, *Microscopy*, 2013, **62**, 81-93.
18. P. Hinterdorfer, M. F. Garcia-Parajo and Y. F. Dufrene, *Accounts Chem. Res.*, 2012, **45**, 327-336.
19. J. Shi, E. S. McLamore and D. M. Porterfield, *Biosens. Bioelectron.*, 2013, **40**, 127-134.
20. P. Happel, D. Thatenhorst and I. D. Dietzel, *Sensors*, 2012, **12**, 14983-15008.
21. J. Rheinlaender and T. E. Schaffer, *J. Appl. Phys.*, 2009, **105**.
22. J. Rheinlaender, N. A. Geisse, R. Proksch and T. E. Schaffer, *Langmuir*, 2011, **27**, 697-704.
23. D. Klenerman, A. Shevchuk, P. Novak, Y. E. Korchev and S. J. Davis, *Philos. Trans. R. Soc. B-Biol. Sci.*, 2013, **368**, 11.
24. V. O. Nikolaev, A. Moshkov, A. R. Lyon, M. Miragoli, P. Novak, H. Paur, M. J. Lohse, Y. E. Korchev, S. E. Harding and J. Gorelik, *Science*, 2010, **327**, 1653-1657.
25. Y. E. Korchev, Y. A. Negulyaev, C. R. W. Edwards, I. Vodyanoy and M. J. Lab, *Nat. Cell Biol.*, 2000, **2**, 616-619.
26. M. J. Lab, A. Bhargava, P. T. Wright and J. Gorelik, *Am. J. Physiol.-Heart Circul. Physiol.*, 2013, **304**, H1-H11.
27. M. Miragoli, A. Moshkov, P. Novak, A. Shevchuk, V. O. Nikolaev, I. El-Hamamsy, C. M. F. Potter, P. Wright, S. Kadir, A. R. Lyon, J. A. Mitchell, A. H. Chester, D.

Klenerman, M. J. Lab, Y. E. Korchev, S. E. Harding and J. Gorelik, *J. R. Soc. Interface*, 2011, **8**, 913-925.
28. C. C. Chen, Y. Zhou and L. A. Baker, in *Annual Review of Analytical Chemistry, Vol 5*, eds. R. G. Cooks and E. S. Yeung, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 5, pp. 207-228.
29. K. Narayan, T. J. Prosa, J. Fu, T. F. Kelly and S. Subramaniam, *J. Struct. Biol.*, 2012, **178**, 98-107.
30. S. Bergner, P. Vatsyayan and F. M. Matysik, *Anal. Chim. Acta*, 2013, **775**, 1-13.
31. I. Beaulieu, S. Kuss, J. Mauzeroll and M. Geissler, *Anal. Chem.*, 2011, **83**, 1485-1492.
32. M. M. N. Zhang, Y. T. Long and Z. F. Ding, *Chemistry Central Journal*, 2012, **6**.
33. S. Amemiya, A. J. Bard, F. R. F. Fan, M. V. Mirkin and P. R. Unwin, in *Annual Review of Analytical Chemistry*, Annual Reviews, Palo Alto, Editon edn., 2008, vol. 1, pp. 95-131.
34. X. C. Zhao, P. M. Diakowski and Z. F. Ding, *Anal. Chem.*, 2010, **82**, 8371-8373.
35. X. C. Zhao, S. Lam, J. Jass and Z. F. Ding, *Electrochem. Commun.*, 2010, **12**, 773-776.
36. G. J. Puppels, F. F. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin and T. M. Jovin, *Nature*, 1990, **347**, 301-303.
37. M. M. Mariani, P. J. R. Day and V. Deckert, *Integr. Biol.*, 2010, **2**, 94-101.
38. C. Krafft, T. Knetschke, R. H. Funk and R. Salzer, *Anal Chem*, 2006, **78**, 4424-4429.
39. M. Okada, N. I. Smith, A. F. Palonpon, H. Endo, S. Kawata, M. Sodeoka and K. Fujita, *Proc Natl Acad Sci U S A*, 2012, **109**, 28-32.
40. I. W. Schie, J. Wu, T. Weeks, M. A. Zern, J. C. Rutledge and T. Huser, *J Biophotonics*, 2011, **4**, 425-434.
41. N. Testerink, M. Ajat, M. Houweling, J. F. Brouwers, V. V. Pully, H. J. van Manen, C. Otto, J. B. Helms and A. B. Vaandrager, *PloS one*, 2012, **7**, e34945.
42. E. Brauchle and K. Schenke-Layland, *Biotechnol. J.*, 2013, **8**, 288-297.
43. C. Krafft, B. Dietzek and J. Popp, *The Analyst*, 2009, **134**, 1046-1057.
44. A. Ramoji, U. Neugebauer, T. Bocklitz, M. Foerster, M. Kiehntopf, M. Bauer and J. Popp, *Anal Chem*, 2012, **84**, 5335-5342.
45. U. Neugebauer, J. H. Clement, T. Bocklitz, C. Krafft and J. Popp, *J. Biophotonics*, 2010, **3**, 579-587.
46. C. Beleites, U. Neugebauer, T. Bocklitz, C. Krafft and J. Popp, *Anal Chim Acta*, 2013, **760**, 25-33.
47. I. W. Schie and T. Huser, *Appl. Spectrosc.*, 2013, **67**, 813-828.
48. K. Brautigam, T. Bocklitz, M. Schmitt, P. Rosch and J. Popp, *Chemphyschem*, 2013, **14**, 550-553.
49. N. Bergner, C. Krafft, K. D. Geiger, M. Kirsch, G. Schackert and J. Popp, *Anal Bioanal Chem*, 2012, **403**, 719-725.
50. M. Q. Li, J. Xu, M. Romero-Gonzalez, S. A. Banwart and W. E. Huang, *Curr. Opin. Biotechnol.*, 2012, **23**, 56-63.
51. S. Dochow, C. Krafft, U. Neugebauer, T. Bocklitz, T. Henkel, G. Mayer, J. Albert and J. Popp, *Lab Chip*, 2011, **11**, 1484-1490.
52. B. R. Wood, P. Caspers, G. J. Puppels, S. Pandiancherri and D. McNaughton, *Anal. Bioanal. Chem.*, 2007, **387**, 1691-1703.
53. C. Matthaus, S. Schubert, M. Schmitt, C. Krafft, B. Dietzek, U. S. Schubert and J. Popp, *Chemphyschem*, 2013, **14**, 155-161.
54. U. Neugebauer, S. H. Heinemann, M. Schmitt and J. Popp, *Anal. Chem.*, 2011, **83**, 344-350.
55. H. Yuan, J. K. Register, Hsin-NengWang, A. M. Fales, Y. Liu and T. Vo-Dinh, *Anal Bioanal Chem*, 2013, **405**, 6165-6180.

56. E. A. Vitol, Z. Orynbayeva, G. Friedman and Y. Gogotsi, *J. Raman Spectrosc.*, 2012, **43**, 817-827.
57. L. Rodriguez-Lorenzo, L. Fabris and R. A. Alvarez-Puebla, *Anal Chim Acta*, 2012, **745**, 10-23.
58. T. Schmid, L. Opilik, C. Blum and R. Zenobi, *Angew. Chem.-Int. Edit.*, 2013, **52**, 5940-5954.
59. L. Quaroni and T. Zlateva, *The Analyst*, 2011, **136**, 3219-3232.
60. P. Lasch, A. Pacifico and M. Diem, *Biopolymers*, 2002, **67**, 335-338.
61. J. M. Schubert, A. I. Mazur, B. Bird, M. Miljkovic and M. Diem, *J Biophotonics*, 2010, **3**, 588-596.
62. S. Boydston-White, M. Romeo, T. Chernenko, A. Regina, M. Miljkovic and M. Diem, *Biochim Biophys Acta*, 2006, **1758**, 908-914.
63. D. R. Whelan, K. R. Bambery, P. Heraud, M. J. Tobin, M. Diem, D. McNaughton and B. R. Wood, *Nucleic Acids Res*, 2011, **39**, 5439-5448.
64. S. G. Kazarian and K. L. A. Chan, *The Analyst*, 2013, **138**, 1940-1951.
65. J. P. Pezacki, J. A. Blake, D. C. Danielson, D. C. Kennedy, R. K. Lyn and R. Singaravelu, *Nat. Chem. Biol.*, 2011, **7**, 137-145.
66. C. Krafft, B. Dietzek, M. Schmitt and J. Popp, *J Biomed Opt*, 2012, **17**, 040801.
67. I. Pope, W. Langbein, P. Borri and P. Watson, *Methods in enzymology*, 2012, **504**, 273-291.
68. A. Folick, W. Min and M. C. Wang, *Curr. Opin. Genet. Dev.*, 2011, **21**, 585-590.
69. L. Wei, Y. Yu, Y. Shen, M. C. Wang and W. Min, *Proc Natl Acad Sci U S A*, 2013, **110**, 11226-11231.
70. L. Sacconi, I. M. Tolic-Norrelykke, M. D'Amico, F. Vanzi, M. Olivotto, R. Antolini and E. S. Pavone, *Cell Biochem. Biophys.*, 2006, **45**, 289-302.
71. A. H. Reshak, V. Sarafis and R. Heintzmann, *Micron*, 2009, **40**, 378-385.
72. P. R. Sander, P. Hau, S. Koch, K. Schutze, U. Bogdahn, H. R. Kalbitzer and L. Aigner, *Trends Biotechnol.*, 2013, **31**, 204-213.
73. S. S. Rubakhin, E. V. Romanova, P. Nemes and J. V. Sweedler, *Nat. Methods*, 2011, **8**, S20-S29.
74. C. E. Neumaier, G. Baio, S. Ferrini, G. Corte and A. Daga, *Tumori*, 2008, **94**, 226-233.
75. J. B. Aguayo, S. J. Blackband, J. Schoeniger, M. A. Mattingly and M. Hintermann, *Nature*, 1986, **322**, 190-191.
76. S. C. Grant, N. R. Aiken, H. D. Plant, S. Gibbs, T. H. Mareci, A. G. Webb and S. J. Blackband, *Magnetic Resonance in Medicine*, 2000, **44**, 19-22.
77. S. C. Lee, J. H. Cho, D. Mietchen, Y. S. Kim, K. S. Hong, C. Lee, D. M. Kang, K. D. Park, B. S. Choi and C. Cheong, *Biophysical journal*, 2006, **90**, 1797-1803.
78. E. J. Lanni, S. S. Rubakhin and J. V. Sweedler, *Journal of Proteomics*, 2012, **75**, 5036-5051.
79. E. R. Amstalden van Hove, D. F. Smith and R. M. Heeren, *Journal of chromatography. A*, 2010, **1217**, 3946-3954.
80. S. Chandra, W. Tjarks, D. R. Lorey and R. F. Barth, *Journal of Microscopy-Oxford*, 2008, **229**, 92-103.
81. L. E. Wedlock, M. R. Kilburn, J. B. Cliff, L. Filgueira, M. Saunders and S. J. Berners-Price, *Metallomics*, 2011, **3**, 917-925.
82. C. Quintana, T. D. Wu, B. Delatour, M. Dhenain, J. L. Guerquin-Kern and A. Croisy, *Microscopy Research and Technique*, 2007, **70**, 281-295.
83. C. Quintana, S. Bellefqih, J. Y. Laval, J. L. Guerquin-Kern, T. D. Wu, J. Avila, I. Ferrer, R. Arranz and C. Patino, *J. Struct. Biol.*, 2006, **153**, 42-54.

84. S. Chandra, in *The Encyclopedia of Mass Spectrometry*, eds. M. L. Gross and R. M. Caprioli, Elsevier, Editon edn., 2010, pp. 469-480.
85. S. Chandra, G. W. Kabalka, D. R. Lorey, D. R. Smith and J. A. Coderre, *Clinical Cancer Research*, 2002, **8**, 2675-2683.
86. G. W. Kabalka, A. L. Shaikh, R. F. Barth, T. Y. Huo, W. L. Yang, P. M. Gordnier and S. Chandra, *Applied Radiation and Isotopes*, 2011, **69**, 1778-1781.
87. J. L. Guerquin-Kern, F. Hillion, J. C. Madelmont, P. Labarre, J. Papon and A. Croisy, *Biomed Eng Online*, 2004, **3**, 10.
88. M. K. Passarelli and N. Winograd, *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 2011, **1811**, 976-990.
89. M. L. Steinhauser, A. P. Bailey, S. E. Senyo, C. Guillermier, T. S. Perlstein, A. P. Gould, R. T. Lee and C. P. Lechene, *Nature*, 2012, **481**, 516-U131.
90. R. Ait-Belkacem, L. Sellami, C. Villard, E. DePauw, D. Calligaris and D. Lafitte, *Trends Biotechnol.*, 2012, **30**, 466-474.
91. J. A. Hankin, R. M. Barkley and R. C. Murphy, *Journal of the American Society for Mass Spectrometry*, 2007, **18**, 1646-1652.
92. E. B. Monroe, J. C. Jurchen, B. A. Koszczuk, J. L. Losh, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2006, **78**, 6826-6832.
93. K. R. Tucker, E. J. Lanni, L. A. Serebryannyy, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2011, **83**, 9181-9185.
94. N. Goto-Inoue, T. Hayasaka, N. Zaima, Y. Kashiwagi, M. Yamamoto, M. Nakamoto and M. Setou, *Journal of the American Society for Mass Spectrometry*, 2010, **21**, 1940-1943.
95. A. F. M. Altelaar, I. M. Taban, L. A. McDonnell, P. Verhaert, R. P. J. de Lange, R. A. H. Adan, W. J. Mooi, R. M. A. Heeren and S. R. Piersma, *International Journal of Mass Spectrometry*, 2007, **260**, 203-211.
96. S. Guenther, A. Rompp, W. Kummer and B. Spengler, *International Journal of Mass Spectrometry*, 2011, **305**, 228-237.
97. T. Harada, A. Yuba-Kubo, Y. Sugiura, N. Zaima, T. Hayasaka, N. Goto-Inoue, M. Wakui, M. Suematsu, K. Takeshita, K. Ogawa, Y. Yoshida and M. Setou, *Anal. Chem.*, 2009, **81**, 9153-9157.
98. M. C. Roy, H. Nakanishi, K. Takahashi, S. Nakanishi, S. Kajihara, T. Hayasaka, M. Setou, K. Ogawa, R. Taguchi and T. Naito, *Journal of Lipid Research*, 2011, **52**, 463-470.
99. B. Spengler and M. Hubert, *Journal of the American Society for Mass Spectrometry*, 2002, **13**, 735-748.
100. W. Bouschen, O. Schulz, D. Eikel and B. Spengler, *Rapid Communications in Mass Spectrometry*, 2010, **24**, 355-364.
101. P. L. Urban, T. Schmid, A. Amantonico and R. Zenobi, *Anal Chem*, 2011, **83**, 1843-1849.
102. B. Shrestha, J. M. Patt and A. Vertes, *Anal Chem*, 2011, **83**, 2947-2955.
103. Q. Zhao, C. H. Huang and F. Y. Li, *Chem. Soc. Rev.*, 2011, **40**, 2508-2524.
104. H. Mattoussi, G. Palui and H. B. Na, *Adv. Drug Deliv. Rev.*, 2012, **64**, 138-166.
105. P. Pierobon and G. Cappello, *Adv Drug Deliv Rev*, 2012, **64**, 167-178.
106. M.-H. Sung, *Cells*, 2013, **2**, 284-293.
107. S. Stehbens, H. Pemble, L. Murrow and T. Wittmann, *Methods in enzymology*, 2012, **504**, 293-313.
108. J. Kind, L. Pagie, H. Ortabozkoyun, S. Boyle, S. S. de Vries, H. Janssen, M. Amendola, L. D. Nolen, W. A. Bickmore and B. van Steensel, *Cell*, 2013, **153**, 178-192.
109. L. Lu, M. S. Ladinsky and T. Kirchhausen, *J Cell Biol*, 2011, **194**, 425-440.

110. A. K. Dunsch, D. Hammond, J. Lloyd, L. Schermelleh, U. Gruneberg and F. A. Barr, *J Cell Biol*, 2012, **198**, 1039-1054.
111. T. Ha, T. Enderle, D. F. Ogletree, D. S. Chemla, P. R. Selvin and S. Weiss, *Proc Natl Acad Sci U S A*, 1996, **93**, 6264-6268.
112. T. Forster, *Modern Quantum Chemistry*, Academic, New York, 1965.
113. S. Zadran, S. Standley, K. Wong, E. Otiniano, A. Amighi and M. Baudry, *Appl Microbiol Biotechnol*, 2012, **96**, 895-902.
114. J. Wiedenmann, F. Oswald and G. U. Nienhaus, *IUBMB Life*, 2009, **61**, 1029-1042.
115. T. Ansbacher, H. K. Srivastava, T. Stein, R. Baer, M. Merkx and A. Shurki, *Phys Chem Chem Phys*, 2012, **14**, 4109-4117.
116. B. R. Waterhouse, M. Gijsen, P. R. Barber, I. D. Tullis, B. Vojnovic and A. Kong, *Oncotarget*, 2011, **2**, 728-736.
117. M. Ouyang, H. Huang, N. C. Shaner, A. G. Remacle, S. A. Shiryaev, A. Y. Strongin, R. Y. Tsien and Y. Wang, *Cancer Res*, 2010, **70**, 2204-2212.
118. M. Willemse, E. Janssen, F. de Lange, B. Wieringa and J. Fransen, *Nat Biotechnol*, 2007, **25**, 170-172.
119. H. Imamura, K. P. Nhat, H. Togawa, K. Saito, R. Iino, Y. Kato-Yamada, T. Nagai and H. Noji, *Proc Natl Acad Sci U S A*, 2009, **106**, 15651-15656.
120. J. W. Borst, M. Willemse, R. Slijkhuis, G. van der Krogt, S. P. Laptenok, K. Jalink, B. Wieringa and J. A. Fransen, *PloS one*, 2010, **5**, e13862.
121. C. Depry, S. Mehta and J. Zhang, *Pflugers Arch*, 2013, **465**, 373-381.
122. D. Geissler, S. Stufler, H. G. Lohmannsroben and N. Hildebrandt, *J Am Chem Soc*, 2013, **135**, 1102-1109.
123. I. Ziomkiewicz, A. Loman, R. Klement, C. Fritsch, A. S. Klymchenko, G. Bunt, T. M. Jovin and D. J. Arndt-Jovin, *Cytometry A*, 2013.
124. W. Becker, *J. Microsc.*. 2012, **247**, 119-136.
125. Y. Sun, R. N. Day and A. Periasamy, *Nat Protoc*, 2011, **6**, 1324-1340.
126. E. A. Reits and J. J. Neefjes, *Nat Cell Biol*, 2001, **3**, E145-147.
127. F. Mueller, D. Mazza, T. J. Stasevich and J. G. McNally, *Curr Opin Cell Biol*, 2010, **22**, 403-411.
128. N. Rainy, D. Chetrit, V. Rouger, H. Vernitsky, O. Rechavi, D. Marguet, I. Goldstein, M. Ehrlich and Y. Kloog, *Cell Death Dis*, 2013, **4**, e726.
129. G. Herbomel, M. Kloster-Landsberg, E. G. Folco, E. Col, Y. Usson, C. Vourc'h, A. Delon and C. Souchier, *PloS one*, 2013, **8**, e67566.
130. K. B. Im, U. Schmidt, M. S. Kang, J. Y. Lee, F. Bestvater and M. Wachsmuth, *Cytometry A*, 2013.
131. E. L. Elson, *Biophysical journal*, 2011, **101**, 2855-2870.
132. P. Brazda, T. Szekeres, B. Bravics, K. Toth, G. Vamosi and L. Nagy, *Journal of cell science*, 2011, **124**, 3631-3642.
133. M. Dong, M. M. Martinez, M. F. Mayer and D. Pappas, *The Analyst*, 2012, **137**, 2997-3003.
134. M. M. Martinez, R. D. Reif and D. Pappas, *Anal Bioanal Chem*, 2010, **396**, 1177-1185.
135. H. Sadamoto, K. Saito, H. Muto, M. Kinjo and E. Ito, *PloS one*, 2011, **6**, e20285.
136. K. Saito, I. Wada, M. Tamura and M. Kinjo, *Biochemical and biophysical research communications*, 2004, **324**, 849-854.
137. A. Sasaki and M. Kinjo, *Journal of controlled release : official journal of the Controlled Release Society*, 2010, **143**, 104-111.
138. K. Bacia, S. A. Kim and P. Schwille, *Nat Methods*, 2006, **3**, 83-89.
139. G. Ball, R. M. Parton, R. S. Hamilton and I. Davis, *Methods in enzymology*, 2012, **504**, 29-55.

140. L. Schermelleh, R. Heintzmann and H. Leonhardt, *J Cell Biol*, 2010, **190**, 165-175.
141. D. C. Jans, C. A. Wurm, D. Riedel, D. Wenzel, F. Stagge, M. Deckers, P. Rehling and S. Jakobs, *Proc Natl Acad Sci U S A*, 2013, **110**, 8936-8941.
142. K. Ramser and D. Hanstorp, *J. Biophotonics*, 2010, **3**, 187-206.
143. F. Opazo, A. Punge, J. Buckers, P. Hoopmann, L. Kastrup, S. W. Hell and S. O. Rizzoli, *Traffic*, 2010, **11**, 800-812.
144. D. Wildanger, R. Medda, L. Kastrup and S. W. Hell, *J Microsc*, 2009, **236**, 35-43.
145. C. A. Wurm, D. Neumann, M. A. Lauterbach, B. Harke, A. Egner, S. W. Hell and S. Jakobs, *Proc Natl Acad Sci U S A*, 2011, **108**, 13546-13551.
146. J. Tonnesen and U. V. Nagerl, *Exp Neurol*, 2013, **242**, 33-40.
147. D. S. Lidke and K. A. Lidke, *Journal of cell science*, 2012, **125**, 2571-2580.
148. M. G. Gustafsson, *J Microsc*, 2000, **198**, 82-87.
149. M. G. L. Gustafsson, *Proc Natl Acad Sci U S A*, 2005, **102**, 13081-13086.
150. R. Heintzmann, T. M. Jovin and C. Cremer, *J. Opt. Soc. Am. A*, 2002, **19**, 1599-1609.
151. D. Baddeley, V. O. Chagin, L. Schermelleh, S. Martin, A. Pombo, P. M. Carlton, A. Gahl, P. Domaing, U. Birk, H. Leonhardt, C. Cremer and M. C. Cardoso, *Nucleic Acids Res*, 2010, **38**, e8.
152. L. Schermelleh, P. M. Carlton, S. Haase, L. Shao, L. Winoto, P. Kner, B. Burke, M. C. Cardoso, D. A. Agard, M. G. Gustafsson, H. Leonhardt and J. W. Sedat, *Science*, 2008, **320**, 1332-1336.
153. Y. Markaki, D. Smeets, S. Fiedler, V. J. Schmid, L. Schermelleh, T. Cremer and M. Cremer, *Bioessays*, 2012, **34**, 412-426.
154. K. A. Lidke, B. Rieger, T. M. Jovin and R. Heintzmann, *Opt Express*, 2005, **13**, 7052-7062.
155. P. Sengupta, S. Van Engelenburg and J. Lippincott-Schwartz, *Developmental cell*, 2012, **23**, 1092-1102.
156. T. Dertinger, R. Colyer, R. Vogel, J. Enderlein and S. Weiss, *Opt Express*, 2010, **18**, 18875-18885.
157. P. Dedecker, G. C. H. Mo, T. Dertinger and J. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 10909-10914.
158. S. Cox, E. Rosten, J. Monypenny, T. Jovanovic-Talisman, D. T. Burnette, J. Lippincott-Schwartz, G. E. Jones and R. Heintzmann, *Nat. Methods*, 2012, **9**, 195-200.
159. J. W. Uhr, M. L. Huebschman, E. P. Frenkel, N. L. Lane, R. Ashfaq, H. Liu, D. R. Rana, L. Cheng, A. T. Lin, G. A. Hughes, X. J. Zhang and H. R. Garner, *Transl Res*, 2012, **159**, 366-375.
160. L. Gao, R. T. Kester, N. Hagen and T. S. Tkaczyk, *Opt Express*, 2010, **18**, 14330-14344.
161. A. D. Elliott, L. Gao, A. Ustione, N. Bedard, R. Kester, D. W. Piston and T. S. Tkaczyk, *Journal of cell science*, 2012, **125**, 4833-4840.
162. S. J. Leavesley, N. Annamdevula, J. Boni, S. Stocker, K. Grant, B. Troyanovsky, T. C. Rich and D. F. Alvarez, *J Biophotonics*, 2012, **5**, 67-84.
163. M. Coelho, N. Maghelli and I. M. Tolic-Norrelykke, *Integr. Biol.*, 2013, **5**, 748-758.
164. R. Iino, I. Koyama and A. Kusumi, *Biophysical journal*, 2001, **80**, 2667-2677.
165. K. Gowrishankar, S. Ghosh, S. Saha, R. C, S. Mayor and M. Rao, *Cell*, 2012, **149**, 1353-1367.
166. K. G. Stenkula, V. A. Lizunov, S. W. Cushman and J. Zimmerberg, *Cell metabolism*, 2010, **12**, 250-259.
167. L. Wang, M. A. Bittner, D. Axelrod and R. W. Holz, *Molecular biology of the cell*, 2008, **19**, 3944-3955.
168. Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii and M. Hide, *Allergol. Int.*, 2013, **62**, 163-169.

169. D. Peelen, V. Kodoyianni, J. Lee, T. Zheng, M. R. Shortreed and L. M. Smith, *Journal of proteome research*, 2006, **5**, 1580-1585.
170. Y. Yanase, T. Hiragun, S. Kaneko, H. J. Gould, M. W. Greaves and M. Hide, *Biosens Bioelectron*, 2010, **26**, 674-681.
171. M. Horii, H. Shinohara, Y. Iribe and M. Suzuki, *The Analyst*, 2011, **136**, 2706-2711.
172. M. West, A. T. Ellis, P. J. Potts, C. Streli, C. Vanhoof, D. Wegrzynek and P. Wobrauschek, *J. Anal. At. Spectrom.*, 2011, **26**, 1919-1963.
173. R. Ortega, *J. Anal. At. Spectrom.*, 2011, **26**, 23-29.
174. C. T. Dillon, *Aust. J. Chem.*, 2012, **65**, 204-217.
175. W. Meyer-Ilse, D. Hamamoto, A. Nair, S. A. Lelievre, G. Denbeaux, L. Johnson, A. L. Pearson, D. Yager, M. A. Legros and C. A. Larabell, *J Microsc*, 2001, **201**, 395-403.
176. S. Ahn, S. Y. Jung and S. J. Lee, *Molecules*, 2013, **18**, 5858-5890.
177. H. N. Chapman and K. A. Nugent, *Nat. Photonics*, 2010, **4**, 833-839.
178. G. McDermott, M. A. Le Gros and C. A. Larabell, in *Annual Review of Physical Chemistry, Vol 63*, eds. M. A. Johnson and T. J. Martinez, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 63, pp. 225-239.
179. G. E. Murphy, K. Narayan, B. C. Lowekamp, L. M. Hartnell, J. A. Heymann, J. Fu and S. Subramaniam, *J Struct Biol*, 2011, **176**, 268-278.
180. Z. Li, L. Q. Chu, J. V. Sweedler and P. W. Bohn, *Anal. Chem.*, 2010, **82**, 2608-2611.
181. S. Yilmaz and A. K. Singh, *Curr. Opin. Biotechnol.*, 2012, **23**, 437-443.
182. R. S. Lasken, *Nat Rev Microbiol*, 2007, **10**, 631-640.
183. H. C. Fan, J. B. Wang, A. Potanina and S. R. Quake, *Nat. Biotechnol.*, 2011, **29**, 51-+.
184. N. Navin, J. Kendall, J. Troge, P. Andrews, L. Rodgers, J. McIndoo, K. Cook, A. Stepansky, D. Levy, D. Esposito, L. Muthuswamy, A. Krasnitz, W. R. McCombie, J. Hicks and M. Wigler, *Nature*, 2011, **472**, 90-U119.
185. M. Malecki and W. Szybalski, *Gene*, 2012, **493**, 132-139.
186. Y. Hou, L. T. Song, P. Zhu, B. Zhang, Y. Tao, X. Xu, F. Q. Li, K. Wu, J. Liang, D. Shao, H. J. Wu, X. F. Ye, C. Ye, R. H. Wu, M. Jian, Y. Chen, W. Xie, R. R. Zhang, L. Chen, X. Liu, X. T. Yao, H. C. Zheng, C. Yu, Q. B. Li, Z. L. Gong, M. Mao, X. Yang, L. Yang, J. X. Li, W. Wang, Z. H. Lu, N. Gu, G. Laurie, L. Bolund, K. Kristiansen, J. Wang, H. M. Yang, Y. R. Li, X. Q. Zhang and J. Wang, *Cell*, 2012, **148**, 873-885.
187. X. Xu, Y. Hou, X. Yin, L. Bao, A. Tang, L. Song, F. Li, S. Tsang, K. Wu, H. Wu, W. He, L. Zeng, M. Xing, R. Wu, H. Jiang, X. Liu, D. Cao, G. Guo, X. Hu, Y. Gui, Z. Li, W. Xie, X. Sun, M. Shi, Z. Cai, B. Wang, M. Zhong, J. Li, Z. Lu, N. Gu, X. Zhang, L. Goodman, L. Bolund, J. Wang, H. Yang, K. Kristiansen, M. Dean, Y. Li and J. Wang, *Cell*, 2012, **148**, 886-895.
188. D. Wang and S. Bodovitz, *Trends Biotechnol*, 2010, **28**, 281-290.
189. J. Tischler and M. A. Surani, *Curr. Opin. Biotechnol.*, 2013, **24**, 69-78.
190. A. Stahlberg and M. Bengtsson, *Methods*, 2010, **50**, 282-288.
191. A. Raj and A. van Oudenaarden, *Cell*, 2008, **135**, 216-226.
192. M. B. Elowitz, A. J. Levine, E. D. Siggia and P. S. Swain, *Science*, 2002, **297**, 1183-1186.
193. J. M. Raser and E. K. O'Shea, *Science*, 2004, **304**, 1811-1814.
194. A. Raj, C. S. Peskin, D. Tranchina, D. Y. Vargas and S. Tyagi, *PLoS biology*, 2006, **4**, e309.
195. A. Stahlberg, M. Kubista and P. Aman, *Expert Review of Molecular Diagnostics*, 2011, **11**, 735-740.
196. J. Eberwine, D. Lovatt, P. Buckley, H. Dueck, C. Francis, T. K. Kim, J. Lee, M. Lee, K. Miyashiro, J. Morris, T. Peritz, T. Schochet, J. Spaethling, J. Y. Sul and J. Kim, *J. R. Soc. Interface*, 2012, **9**, 3165-3183.

197. S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer, *Clin Chem*, 2009, **55**, 611-622.
198. K. A. Heyries, C. Tropini, M. VanInsberghe, C. Doolin, O. I. Petriv, A. Singhal, K. Leung, C. B. Hughesman and C. L. Hansen, *Nat. Methods*, 2011, **8**, 649-U664.
199. J. B. Fan, J. Chen, C. S. April, J. S. Fisher, B. Klotzle, M. Bibikova, F. Kaper, M. Ronaghi, S. Linnarsson, T. Ota, J. Chien, L. C. Laurent, J. F. Loring, S. V. Nisperos, G. Y. Chen and J. F. Zhong, *PloS one*, 2012, **7**, e30794.
200. J. M. Levsky and R. H. Singer, *Journal of cell science*, 2003, **116**, 2833-2838.
201. P. Lichter, T. Cremer, J. Borden, L. Manuelidis and D. C. Ward, *Hum Genet*, 1988, **80**, 224-234.
202. L. Chatre and M. Ricchetti, *Journal of cell science*, 2013, **126**, 914-926.
203. A. M. Femino, F. S. Fay, K. Fogarty and R. H. Singer, *Science*, 1998, **280**, 585-590.
204. S. Itzkovitz and A. van Oudenaarden, *Nat Methods*, 2011, **8**, S12-19.
205. A. Raj, P. van den Bogaard, S. A. Rifkin, A. van Oudenaarden and S. Tyagi, *Nat Methods*, 2008, **5**, 877-879.
206. Y. Buganim, D. A. Faddah, A. W. Cheng, E. Itskovich, S. Markoulaki, K. Ganz, S. L. Klemm, A. van Oudenaarden and R. Jaenisch, *Cell*, 2012, **150**, 1209-1222.
207. S. Itzkovitz, A. Lyubimova, I. C. Blat, M. Maynard, J. van Es, J. Lees, T. Jacks, H. Clevers and A. van Oudenaarden, *Nat Cell Biol*, 2012, **14**, 106-114.
208. J. Lu and A. Tsourkas, *Nucleic Acids Res*, 2009, **37**, e100.
209. C. Larsson, I. Grundberg, O. Soderberg and M. Nilsson, *Nat Methods*, 2010, **7**, 395-397.
210. N. Rosenfeld, J. W. Young, U. Alon, P. S. Swain and M. B. Elowitz, *Science*, 2005, **307**, 1962-1965.
211. J. Selimkhanov, J. Hasty and L. S. Tsimring, *Curr Opin Biotechnol*, 2012, **23**, 34-40.
212. D. Bakstad, A. Adamson, D. G. Spiller and M. R. White, *Curr Opin Biotechnol*, 2012, **23**, 103-109.
213. E. Bertrand, P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer and R. M. Long, *Mol. Cell.*, 1998, **2**, 437-445.
214. S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303-308.
215. J. M. Spaethling and J. H. Eberwine, *Current opinion in pharmacology*, 2013.
216. T. Bartfai, P. T. Buckley and J. Eberwine, *Trends in pharmacological sciences*, 2012, **33**, 9-16.
217. F. Tang, C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B. B. Tuch, A. Siddiqui, K. Lao and M. A. Surani, *Nat Methods*, 2009, **6**, 377-382.
218. T. Kivioja, A. Vaharautio, K. Karlsson, M. Bonke, M. Enge, S. Linnarsson and J. Taipale, *Nat. Methods*, 2012, **9**, 72-U183.
219. Q. F. Wills, K. J. Livak, A. J. Tipping, T. Enver, A. J. Goldson, D. W. Sexton and C. Holmes, *Nat. Biotechnol.*, 2013, **31**, 748-+.
220. M. J. Levesque, P. Ginart, Y. C. Wei and A. Raj, *Nat. Methods*, 2013, **10**, 865-+.
221. M. Wu and A. K. Singh, *Curr Opin Biotechnol*, 2012, **23**, 83-88.
222. A. F. M. Altelaar and A. J. R. Heck, *Current Opinion in Chemical Biology*, 2012, **16**, 206-213.
223. K. He, N. Wang, W. H. Li and X. M. Zhang, *Curr. Opin. Biotechnol.*, 2012, **23**, 120-125.
224. S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2008, **80**, 7128-7136.
225. P. L. Urban, K. Jefimovs, A. Amantonico, S. R. Fagerer, T. Schmid, S. Madler, J. Puigmarti-Luis, N. Goedecke and R. Zenobi, *Lab Chip*, 2010, **10**, 3206-3209.
226. J. S. Mellors, K. Jorabchi, L. M. Smith and J. M. Ramsey, *Anal. Chem.*, 2010, **82**, 967-973.

227. B. Huang, H. K. Wu, D. Bhaya, A. Grossman, S. Granier, B. K. Kobilka and R. N. Zare, *Science*, 2007, **315**, 81-84.
228. Q. H. Shi, L. D. Qin, W. Wei, F. Geng, R. Fan, Y. S. Shin, D. L. Guo, L. Hood, P. S. Mischel and J. R. Heath, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 419-424.
229. C. Ma, R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R. C. Koya, C. C. Liu, G. A. Kwong, C. G. Radu, A. Ribas and J. R. Heath, *Nature medicine*, 2011, **17**, 738-743.
230. C. Ma, R. Fan and M. Elitas, *Frontiers in oncology*, 2013, **3**, 133.
231. J. Choi, K. R. Love, Y. Gong, T. M. Gierahn and J. C. Love, *Anal. Chem.*, 2011, **83**, 6890-6895.
232. A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, M. Novakova, A. J. Demello, R. H. Templer, P. Parker, M. A. A. Neil, O. Ces, P. French, K. R. Willison and D. Klug, *Lab Chip*, 2011, **11**, 1256-1261.
233. A. Salehi-Reyhani, S. Sharma, E. Burgin, M. Barclay, A. Cass, M. A. A. Neil, O. Ces, K. R. Willison and D. R. Klug, *Lab Chip*, 2013, **13**, 2066-2074.
234. A. Amantonico, P. L. Urban and R. Zenobi, *Anal. Bioanal. Chem.*, 2010, **398**, 2493-2504.
235. A. Amantonico, P. L. Urban, S. R. Fagerer, R. M. Balabin and R. Zenobi, *Anal Chem*, 2010, **82**, 7394-7400.
236. E. P. Go, *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, 2010, **5**, 18-30.
237. S. S. Rubakhin, E. J. Lanni and J. V. Sweedler, *Curr. Opin. Biotechnol.*, 2013, **24**, 95-104.
238. A. Amantonico, J. Y. Oh, J. Sobek, M. Heinemann and R. Zenobi, *Angew. Chem.-Int. Edit.*, 2008, **47**, 5382-5385.
239. M. Heinemann and R. Zenobi, *Curr Opin Biotechnol*, 2011, **22**, 26-31.
240. P. Nemes, A. M. Knolhoff, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2011, **83**, 6810-6817.
241. P. Nemes, S. S. Rubakhin, J. T. Aerts and J. V. Sweedler, *Nat. Protoc.*, 2013, **8**, 783-799.
242. M. Lorenzo Tejedor, H. Mizuno, N. Tsuyama, T. Harada and T. Masujima, *Anal Chem*, 2012, **84**, 5221-5228.
243. T. Okajima, *Curr. Pharm. Biotechnol.*, 2012, **13**, 2623-2631.
244. K. Kleparnik, *Electrophoresis*, 2013, **34**, 70-85.
245. O. Ornatsky, D. Bandura, V. Baranov, M. Nitz, M. A. Winnik and S. Tanner, *J. Immunol. Methods*, 2010, **361**, 1-20.
246. S. C. Bendall, E. F. Simonds, P. Qiu, E. A. D. Amir, P. O. Krutzik, R. Finck, R. V. Bruggner, R. Melamed, A. Trejo, O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Pe'er, S. D. Tanner and G. P. Nolan, *Science*, 2011, **332**, 687-696.
247. C. N. Tsang, K. S. Ho, H. Z. Sun and W. T. Chan, *Journal of the American Chemical Society*, 2011, **133**, 7355-7357.
248. Y. Gao, Y. M. Lin, B. C. Zhang, D. X. Zou, M. H. He, B. Dong, W. Hang and B. L. Huang, *Anal. Chem.*, 2013, **85**, 4268-4272.
249. A. Motta, D. Paris and D. Melck, *Anal. Chem.*, 2010, **82**, 2405-2411.
250. A. H. Zhang, H. Sun, S. Qiu and X. J. Wang, *Magn. Reson. Chem.*, 2013, **51**, 549-556.
251. T. Gebregiworgis and R. Powers, *Comb. Chem. High Throughput Screen*, 2012, **15**, 595-610.
252. S. C. Grant, N. R. Aiken, H. D. Plant, S. Gibbs, T. H. Mareci, A. G. Webb and S. J. Blackband, *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*, 2000, **44**, 19-22.

253. S. C. Lee, J. H. Cho, D. Mietchen, Y. S. Kim, K. S. Hong, C. Lee, D. Kang, K. D. Park, B. S. Choi and C. Cheong, *Biophysical journal*, 2006, **90**, 1797-1803.
254. X. Y. Ye, S. S. Rubakhin and J. V. Sweedler, *The Analyst*, 2008, **133**, 423-433.
255. Y. Zheng, J. Nguyen, Y. Wei and Y. Sun, *Lab Chip*, 2013, **13**, 2464-2483.
256. H. Morgan, T. Sun, D. Holmes, S. Gawad and N. G. Green, *J. Phys. D-Appl. Phys.*, 2007, **40**, 61-70.
257. B. J. Kirby, *Micro- and Nanoscale Fluid Mechanics - Transport in Microfluidic Devices*, Cambridge University Press, New York, 2010.
258. T. Sun and H. Morgan, *Microfluid. Nanofluid.*, 2010, **8**, 423-443.
259. K. C. Cheung, M. Di Berardino, G. Schade-Kampmann, M. Hebeisen, A. Pierzchalski, J. Bocsi, A. Mittag and A. Tarnok, *Cytometry Part A*, 2010, **77A**, 648-666.
260. Y. Zheng, E. Shojaei-Baghini, C. Wang and Y. Sun, *Biosens. Bioelectron.*, 2013, **42**, 496-502.
261. X. J. Han, C. van Berkel, J. Gwyer, L. Capretto and H. Morgan, *Anal. Chem.*, 2012, **84**, 1070-1075.
262. C. van Berkel, J. D. Gwyer, S. Deane, N. Green, J. Holloway, V. Hollis and H. Morgan, *Lab Chip*, 2011, **11**, 1249-1255.
263. D. Holmes, D. Pettigrew, C. H. Reccius, J. D. Gwyer, C. van Berkel, J. Holloway, D. E. Davies and H. Morgan, *Lab Chip*, 2009, **9**, 2881-2889.
264. S. B. Cho and H. Thielecke, *Biosens. Bioelectron.*, 2007, **22**, 1764-1768.
265. S. Cho, M. Castellarnau, J. Samitier and H. Thielecke, *Sensors*, 2008, **8**, 1198-1211.
266. C. V. Kurz, H. Buth, A. Sossalla, V. Vermeersch, V. Toncheva, P. Dubruel, E. Schacht and H. Thielecke, *Biosens. Bioelectron.*, 2011, **26**, 3405-3412.
267. D. Mondal, C. Roychaudhuri, L. Das and J. Chatterjee, *Biomed Microdevices*, 2012, **14**, 955-964.
268. S. K. Lee, W. F. Boron and M. D. Parker, *Sensors*, 2013, **13**, 984-1003.
269. M. Lindau, *Biochim. Biophys. Acta-Gen. Subj.*, 2012, **1820**, 1234-1242.
270. G. Minetti, S. Egee, D. Morsdorf, P. Steffen, A. Makhro, C. Achilli, A. Ciana, J. Wang, G. Bouyer, I. Bernhardt, C. Wagner, S. Thomas, A. Bogdanova and L. Kaestner, *Blood Rev.*, 2013, **27**, 91-101.
271. M. Bebarova, *Gen. Physiol. Biophys.*, 2012, **31**, 131-140.
272. C. Grewer, A. Gameiro, T. Mager and K. Fendler, in *Annual Review of Biophysics, Vol 42*, Annual Reviews, Palo Alto, Editon edn., 2013, vol. 42, pp. 95-120.
273. L. Yobas, *J. Micromech. Microeng.*, 2013, **23**, 16.
274. G. S. Wilson and M. A. Johnson, *Chem. Rev.*, 2008, **108**, 2462-2481.
275. B. Reid and M. Zhao, *Commun Integr Biol*, 2011, **4**, 524-527.
276. E. S. McLamore and D. M. Porterfield, *Chem. Soc. Rev.*, 2011, **40**, 5308-5320.
277. D. M. Porterfield, *Biosens. Bioelectron.*, 2007, **22**, 1186-1196.
278. R. I. Dmitriev and D. B. Papkovsky, *Cell. Mol. Life Sci.*, 2012, **69**, 2025-2039.
279. C. F. Wu, B. Bull, K. Christensen and J. McNeill, *Angew. Chem.-Int. Edit.*, 2009, **48**, 2741-2745.
280. M. P. Coogan, J. B. Court, V. L. Gray, A. J. Hayes, S. H. Lloyd, C. O. Millet, S. J. A. Pope and D. Lloyd, *Photochem. Photobiol. Sci.*, 2010, **9**, 103-109.
281. T. C. O'Riordan, A. V. Zhdanov, G. V. Ponomarev and D. B. Papkovsky, *Anal. Chem.*, 2007, **79**, 9414-9419.
282. U. Neugebauer, Y. Pellegrin, M. Devocelle, R. J. Forster, W. Signac, N. Morand and T. E. Keyes, *Chemical Communications*, 2008, 5307-5309.
283. R. I. Dmitriev, A. V. Zhdanov, G. V. Ponomarev, D. V. Yashunski and D. B. Papkovsky, *Anal. Biochem.*, 2010, **398**, 24-33.
284. R. Weigert, M. Sramkova, L. Parente, P. Amornphimoltham and A. Masedunskas, *Histochem Cell Biol*, 2010, **133**, 481-491.

285. T. Terai and T. Nagano, *Pflugers Arch*, 2013, **465**, 347-359.
286. C. Grienberger and A. Konnerth, *Neuron*, 2012, **73**, 862-885.
287. P. Kettunen, *Adv Exp Med Biol*, 2012, **740**, 1039-1071.
288. T. Riemensperger, U. Pech, S. Dipt and A. Fiala, *Biochim Biophys Acta*, 2012, **1820**, 1169-1178.
289. K. K. W. Lo, K. Y. Zhang and S. P. Y. Li, *Eur. J. Inorg. Chem.*, 2011, 3551-3568.
290. M. R. Gill and J. A. Thomas, *Chem. Soc. Rev.*, 2012, **41**, 3179-3192.
291. J. J. Shin and C. J. Loewen, *BMC Biol*, 2011, **9**, 85.
292. J. R. Casey, S. Grinstein and J. Orlowski, *Nat Rev Mol Cell Biol*, 2010, **11**, 50-61.
293. J. Llopis, J. M. McCaffery, A. Miyawaki, M. G. Farquhar and R. Y. Tsien, *Proc Natl Acad Sci U S A*, 1998, **95**, 6803-6808.
294. M. Tantama, Y. P. Hung and G. Yellen, *J Am Chem Soc*, 2011, **133**, 10034-10037.
295. J. Kneipp, H. Kneipp, B. Wittig and K. Kneipp, *J. Phys. Chem. C*, 2010, **114**, 7421-7426.
296. R. Barer, *Nature*, 1953, **172**, 1097-1098.
297. N. Lue, W. Choi, G. Popescu, Z. Yaqoob, K. Badizadegan, R. R. Dasari and M. S. Feld, *J Phys Chem A*, 2009, **113**, 13327-13330.
298. W. Choi, C. Fang-Yen, K. Badizadegan, S. Oh, N. Lue, R. R. Dasari and M. S. Feld, *Nat Methods*, 2007, **4**, 717-719.
299. Y. Sung, W. Choi, N. Lue, R. R. Dasari and Z. Yaqoob, *PloS one*, 2012, **7**, e49502.
300. J. Reed, J. Chun, T. A. Zangle, S. Kalim, J. S. Hong, S. E. Pefley, X. Zheng, J. K. Gimzewski and M. A. Teitell, *Biophysical journal*, 2011, **101**, 1025-1031.
301. M. Mir, Z. Wang, Z. Shen, M. Bednarz, R. Bashir, I. Golding, S. G. Prasanth and G. Popescu, *Proc Natl Acad Sci U S A*, 2011, **108**, 13124-13129.
302. Z. Wang, L. Millet, M. Mir, H. Ding, S. Unarunotai, J. Rogers, M. U. Gillette and G. Popescu, *Opt Express*, 2011, **19**, 1016-1026.
303. T. P. Burg, M. Godin, S. M. Knudsen, W. Shen, G. Carlson, J. S. Foster, K. Babcock and S. R. Manalis, *Nature*, 2007, **446**, 1066-1069.
304. M. Godin, F. F. Delgado, S. M. Son, W. H. Grover, A. K. Bryan, A. Tzur, P. Jorgensen, K. Payer, A. D. Grossman, M. W. Kirschner and S. R. Manalis, *Nat. Methods*, 2010, **7**, 387-U370.
305. J. J. Li and K. D. Zhu, *Physics Reports-Review Section of Physics Letters*, 2013, **525**, 223-254.
306. W. H. Grover, A. K. Bryan, M. Diez-Silva, S. Suresh, J. M. Higgins and S. R. Manalis, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 10992-10996.
307. D. Kirmizis and S. Logothetidis, *Int. J. Nanomed.*, 2010, **5**, 137-145.
308. Y. J. Li, C. Wen, H. M. Xie, A. P. Ye and Y. J. Yin, *Colloid Surf. B-Biointerfaces*, 2009, **70**, 169-173.
309. C. T. Lim, E. H. Zhou and S. T. Quek, *J. Biomech.*, 2006, **39**, 195-216.
310. E. H. Zhou, S. T. Quek and C. T. Lim, *Biomech. Model. Mechanobiol.*, 2010, **9**, 563-572.
311. D. H. Kim, P. K. Wong, J. Park, A. Levchenko and Y. Sun, in *Annual Review of Biomedical Engineering*, Annual Reviews, Palo Alto, Editon edn., 2009, vol. 11, pp. 203-233.
312. O. Loh, A. Vaziri and H. Espinosa, *Exp. Mech.*, 2009, **49**, 105-124.
313. M. Lekka, K. Pogoda, J. Gostek, O. Klymenko, S. Prauzner-Bechcicki, J. Wiltowska-Zuber, J. Jaczewska, J. Lekki and Z. Stachura, *Micron*, 2012, **43**, 1259-1266.
314. I. Sraj, C. D. Eggleton, R. Jimenez, E. Hoover, J. Squier, J. Chichester and D. W. M. Marr, *J. Biomed. Opt.*, 2010, **15**.
315. P. C. Ashok and K. Dholakia, *Curr Opin Biotechnol*, 2012, **23**, 16-21.

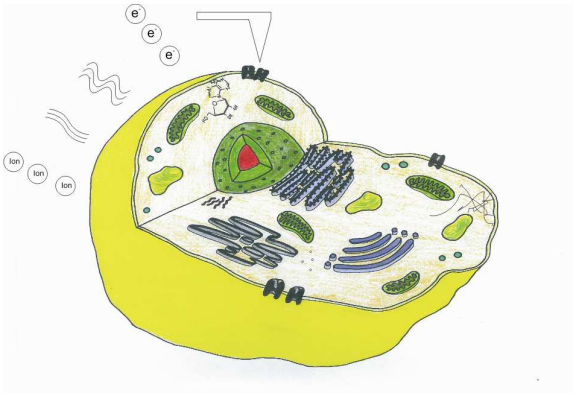
316. J. M. A. Mauritz, T. Tiffert, R. Seear, F. Lautenschlager, A. Esposito, V. L. Lew, J. Guck and C. F. Kaminski, *J. Biomed. Opt.*, 2010, **15**.
317. D. R. Gossett, H. T. K. Tse, S. A. Lee, Y. Ying, A. G. Lindgren, O. O. Yang, J. Y. Rao, A. T. Clark and D. Di Carlo, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 7630-7635.
318. D. Di Carlo, *Jala*, 2012, **17**, 32-42.
319. Y. Zheng and Y. Sun, *Micro & Nano Letters*, 2011, **6**, 327-331.
320. G. S. Du, A. Ravetto, Q. Fang and J. M. J. den Toonder, *Biomedical Microdevices*, 2011, **13**, 29-40.
321. Y. C. Kim, S. J. Park and J. K. Park, *The Analyst*, 2008, **133**, 1432-1439.
322. F. A. Carvalho and N. C. Santos, *IUBMB Life*, 2012, **64**, 465-472.
323. V. Fernandez-Duenas, J. Llorente, J. Gandia, D. O. Borroto-Escuela, L. F. Agnati, C. I. Tasca, K. Fuxe and F. Ciruela, *Methods*, 2012, **57**, 467-472.
324. D. G. Spiller, C. D. Wood, D. A. Rand and M. R. White, *Nature*, 2010, **465**, 736-745.
325. J. Huang, C. Meyer and C. Zhu, *Mol Immunol*, 2012, **52**, 155-164.
326. B. Koos, L. Andersson, C. M. Clausson, K. Grannas, A. Klaesson, G. Cane and O. Soderberg, *Curr Top Microbiol Immunol*, 2013.
327. O. Soderberg, M. Gullberg, M. Jarvius, K. Ridderstrale, K. J. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L. G. Larsson and U. Landegren, *Nat Methods*, 2006, **3**, 995-1000.
328. O. Soderberg, K. J. Leuchowius, M. Gullberg, M. Jarvius, I. Weibrecht, L. G. Larsson and U. Landegren, *Methods*, 2008, **45**, 227-232.
329. M. Blazek, C. Betz, M. Reth, M. N. Hall, R. Zengerle and M. Meier, *Mol Cell Proteomics*, 2013.
330. Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii and M. Hide, *Allergology international : official journal of the Japanese Society of Allergology*, 2013, **62**, 163-169.
331. D. H. M. Dam, J. H. Lee, P. N. Sisco, D. T. Co, M. Zhang, M. R. Wasielewski and T. W. Odom, *ACS Nano*, 2012, **6**, 3318-3326.
332. Z. Feng, W. Zhang, J. Xu, C. Gauron, B. Ducos, S. Vriz, M. Volovitch, L. Jullien, S. Weiss and D. Bensimon, *Rep Prog Phys*, 2013, **76**, 072601.
333. D. Oron, E. Papagiakoumou, F. Anselmi and V. Emiliani, in *Optogenetics: Tools for Controlling and Monitoring Neuronal Activity*, Elsevier Science Bv, Amsterdam, Editon edn., 2012, vol. 196, pp. 119-143.
334. R. H. W. Lam, Y. B. Sun, W. Q. Chen and J. P. Fu, *Lab Chip*, 2012, **12**, 1865-1873.
335. N. J. Sniadecki, A. Anguelouch, M. T. Yang, C. M. Lamb, Z. Liu, S. B. Kirschner, Y. Liu, D. H. Reich and C. S. Chen, *Proc Natl Acad Sci U S A*, 2007, **104**, 14553-14558.
336. S. K. Yoo, P. Y. Lam, M. R. Eichelberg, L. Zasadil, W. M. Bement and A. Huttenlocher, *Journal of cell science*, 2012, **125**, 5702-5710.
337. X. Liu, F. Long, H. Peng, S. J. Aerni, M. Jiang, A. Sanchez-Blanco, J. I. Murray, E. Preston, B. Mericle, S. Batzoglou, E. W. Myers and S. K. Kim, *Cell*, 2009, **139**, 623-633.
338. I. Topalidou, A. van Oudenaarden and M. Chalfie, *Proc Natl Acad Sci U S A*, 2011, **108**, 4063-4068.
339. M. Weber and J. Huisken, *Curr Opin Genet Dev*, 2011, **21**, 566-572.
340. Y. Wu, A. Ghitani, R. Christensen, A. Santella, Z. Du, G. Rondeau, Z. Bao, D. Colon-Ramos and H. Shroff, *Proc Natl Acad Sci U S A*, 2011, **108**, 17708-17713.
341. U. Krzic, S. Gunther, T. E. Saunders, S. J. Streichan and L. Hufnagel, *Nat Methods*, 2012, **9**, 730-733.
342. L. Gao, L. Shao, C. D. Higgins, J. S. Poulton, M. Peifer, M. W. Davidson, X. Wu, B. Goldstein and E. Betzig, *Cell*, 2012, **151**, 1370-1385.

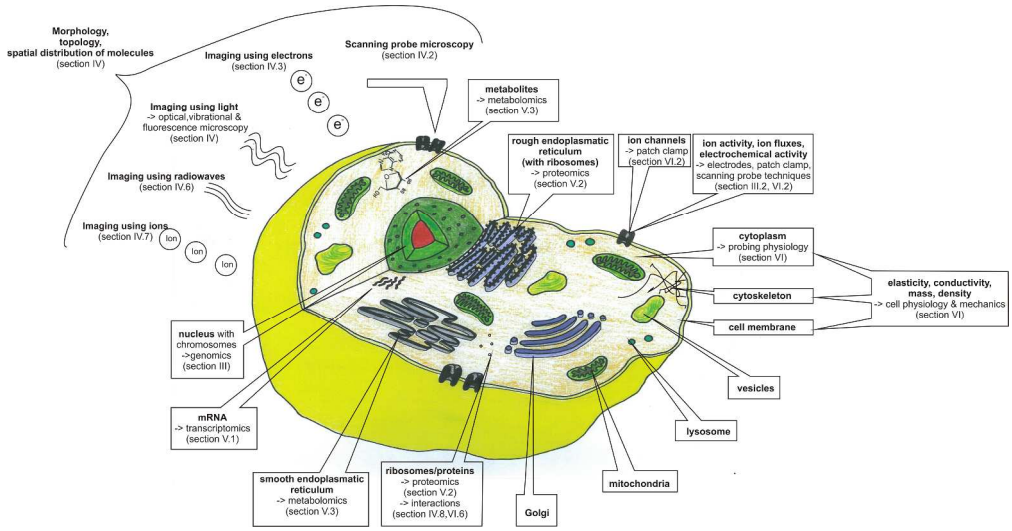
343. F. Progatzky, M. J. Dallman and C. Lo Celso, *Interface focus*, 2013, **3**, 20130001.
344. W. Denk, J. H. Strickler and W. W. Webb, *Science*, 1990, **248**, 73-76.
345. N. R. Bhakta and R. S. Lewis, *Seminars in immunology*, 2005, **17**, 411-420.
346. M. D. Cahalan, I. Parker, S. H. Wei and M. J. Miller, *Nature reviews. Immunology*, 2002, **2**, 872-880.
347. M. Mues, I. Bartholomaus, T. Thestrup, O. Griesbeck, H. Wekerle, N. Kawakami and G. Krishnamoorthy, *Nature medicine*, 2013, **19**, 778-783.
348. L. V. Wang, *Nat Photonics*, 2009, **3**, 503-509.
349. L. Wang, K. Maslov and L. V. Wang, *Proc Natl Acad Sci U S A*, 2013, **110**, 5759-5764.
350. C. Q. Yi, C. W. Li, S. L. Ji and M. S. Yang, *Anal. Chim. Acta*, 2006, **560**, 1-23.
351. S. Lindstrom and H. Andersson-Svahn, *Lab Chip*, 2010, **10**, 3363-3372.
352. J. R. Moffitt, Y. R. Chemla, S. B. Smith and C. Bustamante, in *Annual Review of Biochemistry*, Annual Reviews, Palo Alto, Editon edn., 2008, vol. 77, pp. 205-228.
353. H. Zhang and K. K. Liu, *J. R. Soc. Interface*, 2008, **5**, 671-690.
354. U. Neugebauer, T. Bocklitz, J. H. Clement, C. Krafft and J. Popp, *The Analyst*, 2010, **135**, 3178-3182.
355. B. Kemper, P. Langehanenberg, A. Hoink, G. von Bally, F. Wottowah, S. Schinkinger, J. Guck, J. Kas, I. Bredebusch, J. Schnekenburger and K. Schutze, *J. Biophotonics*, 2010, **3**, 425-431.
356. M. Gyger, D. Rose, R. Stange, T. Kiessling, M. Zink, B. Fabry and J. A. Kas, *Opt. Express*, 2011, **19**, 19212-19222.
357. A. Farre, C. Lopez-Quesada, J. Andilla, E. Martin-Badosa and M. Montes-Usategui, *Opt. Eng.*, 2010, **49**, 6.
358. E. D'Este, G. Baj, P. Beuzer, E. Ferrari, G. Pinato, E. Tongiorgi and D. Cojoc, *Integr. Biol.*, 2011, **3**, 568-577.
359. L. B. Oddershede, *Nat. Chem. Biol.*, 2012, **8**, 879-886.
360. H. M. K. Wong, M. Righini, J. C. Gates, P. G. R. Smith, V. Pruneri and R. Quidant, *Appl. Phys. Lett.*, 2011, **99**, 3.
361. M. L. Juan, M. Righini and R. Quidant, *Nat. Photonics*, 2011, **5**, 349-356.
362. M. Righini, G. Volpe, C. Girard, D. Petrov and R. Quidant, *Phys. Rev. Lett.*, 2008, **100**.
363. A. N. Grigorenko, N. W. Roberts, M. R. Dickinson and Y. Zhang, *Nat. Photonics*, 2008, **2**, 365-370.
364. A. E. Christakou, M. Ohlin, B. Vanherberghen, M. A. Khorshidi, N. Kadri, T. Frisk, M. Wiklund and B. Onfelt, *Integr Biol (Camb)*, 2013, **5**, 712-719.
365. B. Vanherberghen, O. Manneberg, A. Christakou, T. Frisk, M. Ohlin, H. M. Hertz, B. Onfelt and M. Wiklund, *Lab Chip*, 2010, **10**, 2727-2732.
366. M. Ohlin, A. E. Christakou, T. Frisk, B. Onfelt and M. Wiklund, *J. Micromech. Microeng.*, 2013, **23**.
367. I. De Vlaminck and C. Dekker, in *Annual Review of Biophysics, Vol 41*, ed. D. C. Rees, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 41, pp. 453-472.
368. T. Shimada, W. Watanabe, S. Matsunaga, T. Higashi, H. Ishii, K. Fukui, K. Isobe and K. Itoh, *Opt Express*, 2005, **13**, 9869-9880.
369. R. B. Brown and J. Audet, *J. R. Soc. Interface*, 2008, **5**, S131-S138.
370. Y. Q. Lin, R. Trouillon, G. Safina and A. G. Ewing, *Anal. Chem.*, 2011, **83**, 4369-4392.
371. D. C. Essaka, J. Prendergast, R. B. Keithley, M. M. Palcic, O. Hindsgaul, R. L. Schnaar and N. J. Dovichi, *Anal. Chem.*, 2012, **84**, 2799-2804.
372. C. Cecala and J. V. Sweedler, *The Analyst*, 2012, **137**, 2922-2929.

373. V. Lecault, A. K. White, A. Singhal and C. L. Hansen, *Curr Opin Chem Biol*, 2012, **16**, 381-390.
374. R. L. Fernandes, M. Nierychlo, L. Lundin, A. E. Pedersen, P. E. P. Tellez, A. Dutta, M. Carlquist, A. Bolic, D. Schapper, A. C. Brunetti, S. Helmark, A. L. Heins, A. D. Jensen, I. Nopens, K. Rottwitt, N. Szita, J. D. van Elsas, P. H. Nielsen, J. Martinussen, S. J. Sorensen, A. E. Lantz and K. V. Gernaey, *Biotechnol. Adv.*, 2011, **29**, 575-599.
375. J. R. S. Newman, S. Ghaemmaghami, J. Ihmels, D. K. Breslow, M. Noble, J. L. DeRisi and J. S. Weissman, *Nature*, 2006, **441**, 840-846.
376. G. W. Osborne, in *Recent Advances in Cytometry, Part A: Instrumentation, Methods, Fifth Edition*, eds. Z. Darzynkiewicz, E. Holden, A. Orfao, W. Telford and D. Wlodkowic, Editon edn., 2011, vol. 102, pp. 533-556.
377. S. C. Bendall, G. P. Nolan, M. Roederer and P. K. Chattopadhyay, *Trends in Immunology*, 2012, **33**, 323-332.
378. R. N. Zare and S. Kim, in *Annual Review of Biomedical Engineering, Vol 12*, Annual Reviews, Palo Alto, Editon edn., 2010, vol. 12, pp. 187-201.
379. H. Yun, K. Kim and W. G. Lee, *Biofabrication*, 2013, **5**.
380. X. Mu, W. F. Zheng, J. S. Sun, W. Zhang and X. Y. Jiang, *Small*, 2013, **9**, 9-21.
381. J. B. Wang, H. C. Fan, B. Behr and S. R. Quake, *Cell*, 2012, **150**, 402-412.
382. J. Sun, M. D. Masterman-Smith, N. A. Graham, J. Jiao, J. Mottahedeh, D. R. Laks, M. Ohashi, J. DeJesus, K. Kamei, K. B. Lee, H. Wang, Z. T. F. Yu, Y. T. Lu, S. A. Hou, K. Y. Li, M. Liu, N. G. Zhang, S. T. Wang, B. Angenieux, E. Panosyan, E. R. Samuels, J. Park, D. Williams, V. Konkankit, D. Nathanson, R. M. van Dam, M. E. Phelps, H. Wu, L. M. Liau, P. S. Mischel, J. A. Lazareff, H. I. Kornblum, W. H. Yong, T. G. Graeber and H. R. Tseng, *Cancer Res.*, 2010, **70**, 6128-6138.
383. Y. Wang, Z. Z. Chen and Q. L. Li, *Microchimica Acta*, 2010, **168**, 177-195.
384. A.-K. Gustavsson, D. D. v. Niekerk, C. B. Adiels, F. B. d. Preez, M. Goksör and J. L. Snoep, *Febs J.*, 2012, **279**, 2837-2847.
385. M. Zagnoni and J. M. Cooper, in *Recent Advances in Cytometry, Part A: Instrumentation, Methods, Fifth Edition*, eds. Z. Darzynkiewicz, E. Holden, A. Orfao, W. Telford and D. Wlodkowic, Editon edn., 2011, vol. 102, pp. 25-48.
386. T. P. Lagus and J. F. Edd, *J. Phys. D-Appl. Phys.*, 2013, **46**.
387. A. J. Mach, O. B. Adeyiga and D. Di Carlo, *Lab Chip*, 2013, **13**, 1011-1026.
388. A. K. White, M. VanInsberghe, O. I. Petriv, M. Hamidi, D. Sikorski, M. A. Marra, J. Piret, S. Aparicio and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13999-14004.
389. K. Yasuda, A. Hattori, H. Kim, H. Terazono, M. Hayashi, H. Takei, T. Kaneko and F. Nomura, *Microfluid. Nanofluid.*, 2013, **14**, 907-931.
390. H. Yin and D. Marshall, *Curr Opin Biotechnol*, 2012, **23**, 110-119.
391. D. Desmaele, M. Boukallel and S. Regnier, *J Biomech*, 2011, **44**, 1433-1446.
392. M. Ni, W. H. Tong, D. Choudhury, N. A. A. Rahim, C. Iliescu and H. Yu, *Int. J. Mol. Sci.*, 2009, **10**, 5411-5441.
393. R. Fior, S. Maggiolino, M. Lazzarino and O. Sbaizero, *Microsyst. Technol.*, 2011, **17**, 1581-1587.
394. J. Castillo, M. Dimaki and W. E. Svendsen, *Integr. Biol.*, 2009, **1**, 30-42.
395. L. A. Devriese, A. J. Bosma, M. M. van de Heuvel, W. Heemsbergen, E. E. Voest and J. H. Schellens, *Lung cancer*, 2012, **75**, 242-247.
396. T. Xu, C. T. Shu, E. Purdom, D. Dang, D. Ilsley, Y. Guo, J. Weber, S. P. Holmes and P. P. Lee, *Cancer Res*, 2004, **64**, 3661-3667.
397. W. J. Choi, D. I. Jeon, S. G. Ahn, J. H. Yoon, S. Kim and B. H. Lee, *Opt. Express*, 2010, **18**, 23285-23295.

398. B. Vanherberghen, P. E. Olofsson, E. Forslund, M. Sternberg-Simon, M. A. Khorshidi, S. Pacouret, K. Guldevall, M. Enqvist, K. J. Malmberg, R. Mehr and B. Onfelt, *Blood*, 2013, **121**, 1326-1334.
399. A. Stahlberg, D. Andersson, J. Aurelius, M. Faiz, M. Pekna, M. Kubista and M. Pekny, *Nucleic Acids Research*, 2011, **39**.
400. J. Ji, F. Yu, Q. Ji, Z. Li, K. Wang, J. Zhang, J. Lu, L. Chen, Q. E, Y. Zeng and Y. Ji, *Hepatology*, 2012, **56**, 332-349.
401. S. I. Kim and H. I. Jung, *J. Breast Canc.*, 2010, **13**, 125-131.
402. L. Yu, S. R. Ng, Y. Xu, H. Dong, Y. J. Wang and C. M. Li, *Lab Chip*, 2013, **13**, 3163-3182.
403. J. Chen, J. Li and Y. Sun, *Lab Chip*, 2012, **12**, 1753-1767.
404. H. Choi, K. B. Kim, C. S. Jeon, I. Hwang, S. Lee, H. K. Kim, H. C. Kim and T. D. Chung, *Lab Chip*, 2013, **13**, 970-977.
405. K. R. Willison and D. R. Klug, *Curr Opin Biotechnol*, 2013, **24**, 745-751.
406. P. Dalerba, T. Kalisky, D. Sahoo, P. S. Rajendran, M. E. Rothenberg, A. A. Leyrat, S. Sim, J. Okamoto, D. M. Johnston, D. L. Qian, M. Zabala, J. Bueno, N. F. Neff, J. B. Wang, A. A. Shelton, B. Visser, S. Hisamori, Y. Shimono, M. van de Wetering, H. Clevers, M. F. Clarke and S. R. Quake, *Nat. Biotechnol.*, 2011, **29**, 1120-U1111.
407. D. Ramskold, S. Luo, Y. C. Wang, R. Li, Q. Deng, O. R. Faridani, G. A. Daniels, I. Khrebtukova, J. F. Loring, L. C. Laurent, G. P. Schroth and R. Sandberg, *Nat Biotechnol*, 2012, **30**, 777-782.
408. S. C. Bendall and G. P. Nolan, *Nat. Biotechnol.*, 2012, **30**, 639-647.
409. S. Esumi, S. X. Wu, Y. Yanagawa, K. Obata, Y. Sugimoto and N. Tamamaki, *Neuroscience research*, 2008, **60**, 439-451.
410. A. Kawaguchi, T. Ikawa, T. Kasukawa, H. R. Ueda, K. Kurimoto, M. Saitou and F. Matsuzaki, *Development*, 2008, **135**, 3113-3124.
411. J. M. Trimarchi, M. B. Stadler and C. L. Cepko, *PloS one*, 2008, **3**, e1588.
412. R. Lu, N. F. Neff, S. R. Quake and I. L. Weissman, *Nat. Biotechnol.*, 2011, **29**, 928-U229.
413. O. I. Petriv, F. Kuchenbauer, A. D. Delaney, V. Lecault, A. White, D. Kent, L. Marmolejo, M. Heuser, T. Berg, M. Copley, J. Ruschmann, S. Sekulovic, C. Benz, E. Kuroda, V. Ho, F. Antignano, T. Halim, V. Giambra, G. Krystal, C. J. F. Takei, A. P. Weng, J. Piret, C. Eaves, M. A. Marra, R. K. Humphries and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 15443-15448.
414. L. Z. Shi, J. M. Nascimento, C. Chandsawangbhuwana, E. L. Botvinick and M. W. Berns, *Biomed Microdevices*, 2008, **10**, 573-583.
415. M. Iskar, G. Zeller, X. M. Zhao, V. van Noort and P. Bork, *Curr Opin Biotechnol*, 2012, **23**, 609-616.
416. L. Flatz, R. Roychoudhuri, M. Honda, A. Filali-Mouhim, J. P. Goulet, N. Kettaf, M. Lin, M. Roederer, E. K. Haddad, R. P. Sekaly and G. J. Nabel, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 5724-5729.
417. M. T. Dieterlen, K. Eberhardt, A. Tarnok, H. B. Bittner and M. J. Barten, *Methods Cell Biol*, 2011, **103**, 267-284.
418. M. A. Khorshidi, B. Vanherberghen, J. M. Kowalewski, K. R. Garrod, S. Lindstrom, H. Andersson-Svahn, H. Brismar, M. D. Cahalan and B. Onfelt, *Integr. Biol.*, 2011, **3**, 770-778.
419. M. Walde, J. Moneypenny, R. Heintzmann, G. E. Jones and S. Cox, *PloS one*, under review.

All aspects of the characterisation of single cells are reviewed: from morphology to genetics and different omics-techniques to physiological, mechanical and electrical methods, including microfluidics and applications.





Scheme of a single cell with its components (drawn not to scale) and an overview of the techniques that can be used to investigate and characterize the cell.

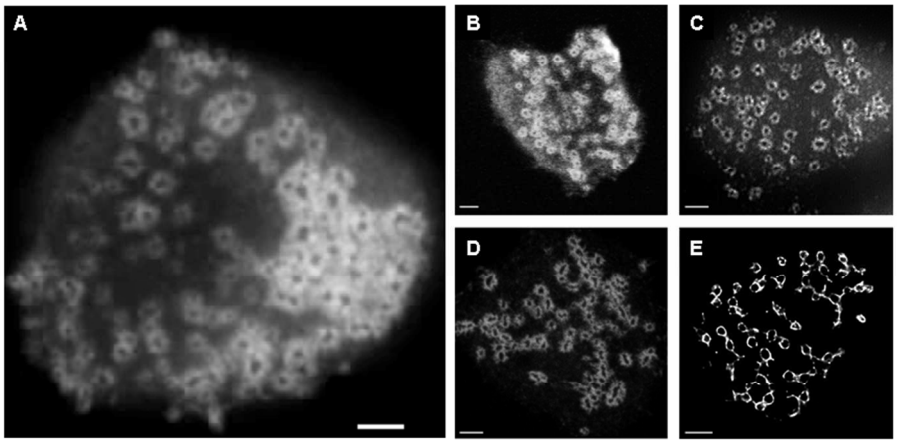
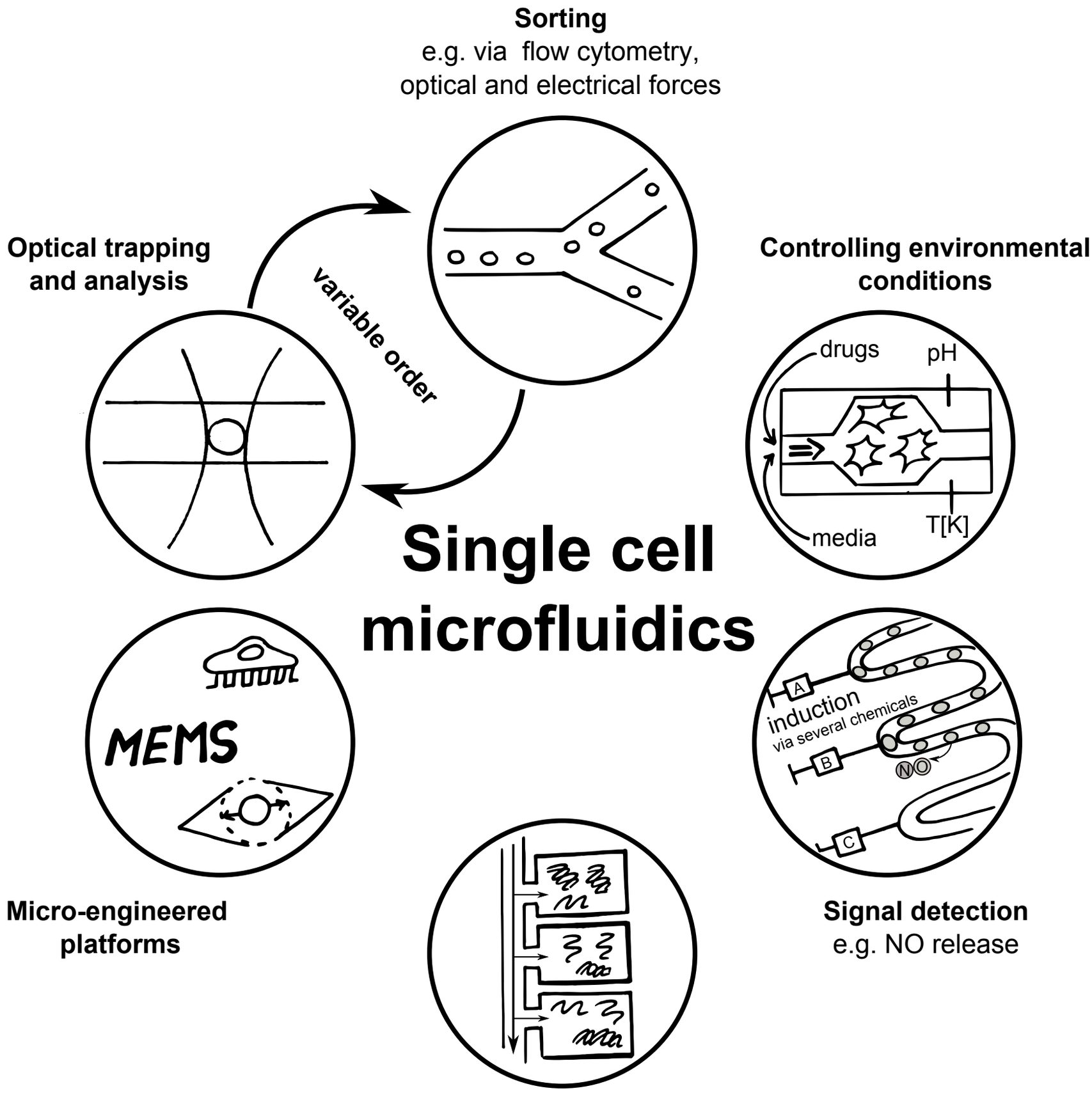


Illustration of the spatial resolution of the different microscopy techniques using the example of the podosome ring of actin associated proteins in THP-1 cells (macrophages). The membrane-cytoskeletal protein vinculin was labelled with Alexa488 (A-C, E) or ATTO-647 (D) for visualization. All scale bars are 2 µm. (Figure adapted from Walde et al. 419 and Cox et al 158).
A: Widefield image of a macrophage. The cylindrical actin-rich podosomes (0.5-2µm in diameter) can be seen as bright circles against a blur background. (section III.8)
B: Confocal image, as the podosomes are very thin structures with only minimal depth profile (they form a flat sample with all the podosomes close to the cover slide surface) the sectioning difference between widefield (A) and confocal (B) is relatively small. (section III.8.1)
C: SIM image. (section III.8.4)
D: STED image recorded on a commercial instrument. Even better resolution can be achieved with home-build STED systems. (section III.8.4)
E: 3B image. No background fluorescence interferes with the signal from the podosomes. (section III.8.4)

254x190mm (96 x 96 DPI)



Making a big thing of a small cell – recent advances in single cell analysis

Kerstin Galler [1,2], Katharina Bräutigam [2,3], Christina Große [1,2], Jürgen Popp [1,2,3], Ute Neugebauer [1,2]

[1] Integrated Research and Treatment Center "Center for Sepsis Control and Care", Jena University Hospital, Erlanger Allee 101, 07747 Jena, Germany

[2] Leibniz Institute of Photonic Technology Jena, Albert-Einstein-Straße 9, 07745 Jena, Germany

[3] Institute of Physical Chemistry and Abbe Center of Photonics, Friedrich Schiller University, Helmholtzweg 4, 07743 Jena, Germany

Abstract

Single cell analysis is an emerging field requiring a high level interdisciplinary collaboration to provide detailed insights into the complex organisation, function and heterogeneity of life. This review is addressed to life science researchers as well as researchers developing novel technologies. It covers all aspects of the characterisation of single cells (with a special focus on mammalian cells) from morphology to genetics and different omics-techniques to physiological, mechanical and electrical methods. In recent years, tremendous advances have been achieved in all fields of single cell analysis: 1. improved spatial and temporal resolution of imaging techniques to enable the tracking of single molecule dynamics within single cells; 2. increased throughput to reveal unexpected heterogeneity between different individual cells raising the question what characterizes a cell type and what is just natural biological variation; and 3. emerging multimodal approaches try to bring together information from complementary techniques paving the way for a deeper understanding of the complexity of biological processes. This review also covers the first successful translations of single cell analysis methods to diagnostic applications in the field of tumour research (especially circulating tumour cells), regenerative medicine, drug discovery and immunology.

Keywords

Imaging, scanning probe microscopy, electron microscopy, Raman, NMR, mass spectrometry, fluorescence, super-resolution microscopy, TIRFM, ~omics (genomics, transcriptomics, proteomics, metabolomics), sequencing, , patch clamp, interaction, signalling, cell type, micromanipulation, trapping, sorting, microfluidics, lab-on-a-chip, AFM, SICM, APT, SECM, FRET, FLIM, FRAP, FCS

Content

I. Introduction: Motivation for single cell analysis

Eukaryotic cells may spend their lives as single individuals; however they have evolved to cooperate closely to form tissues and whole organisms, plants or animals. Conversely, single cell analysis often focuses on individual cells split apart from their tissue of origin. Despite the fact, that this is an artificial situation, single cell investigations gain a special eligibility in raising awareness for small, but substantial differences of single cells in a population of apparently identical cells. Furthermore, single cell investigations play an important role in unravelling mechanistic details of intracellular processes. When we study single cells and try to understand how they function as a biological system we can learn about biological system development, their growth and specialization as well as evolution of single cells and whole organisms. If we can understand biological phenomena at the molecular level, we might be able to understand how subtle differences in cellular phenotype induce biological phenomena such as learning and memory or how cell-specific changes lead to dysfunction and disease states, e.g., in cancer. In order to develop novel therapeutic interventions in response to single cell behaviour, it is important to understand the relationship between biological heterogeneity and signalling pathways. These might involve rare but important events as well as rare cell types, such as stem cells or progenitor cells. Quite often this can lead to a range of questions, for example: 1. what are the important features of a cell? 2. which properties determine the cell type? 3. are the observed differences between two individual cells just the natural cellular variability? 4. are these two similar appearing cells already destined to move in different developmental directions? and 5. how large have the differences between two cells to be in order to assign them to a different cell type? A vast variety of analytical methods and approaches have been developed to study single cells in order to probe their morphological and physiological characteristics, gain molecular, genomic, transcriptomic and quantitative biochemistry information and even monitor their dynamic changes under simulated close-to-*in vivo* conditions. Also, within fully functional organisms, single cells can be identified and tracked which shall ultimately allow studying single cells in their natural, unperturbed environment.

A challenge for single cell studies is the small size of a single cell. Analytical techniques therefore must be able to work with very little sample input. Nevertheless, in order to achieve enough data for significant statistics, they should manage high throughput, and of course – to be affordable in many laboratories – they should not be expensive. In the last few years tremendous advances could be achieved in all fields of single cell analysis. In this review, we

will present the techniques that are currently available for single cell analysis and highlight a few of those recent developments. A wide range of reviews already exists for the different techniques. This review article is not an exhaustive review citing all available references but shall give an overview of the possibilities and maybe create some interest among researchers to try another technique to gain complementary information for their question of interest. Cited review articles and a few original articles shall provide further information for the interested reader. The review is focused on the analysis of mammalian cells, leaving out most of the exciting work done with other eukaryotic cells (such as yeast) or studies on prokaryotic cells. Furthermore, for detailed isolation techniques to retrieve single cells the reader is referred to other recent reviews covering this topic.

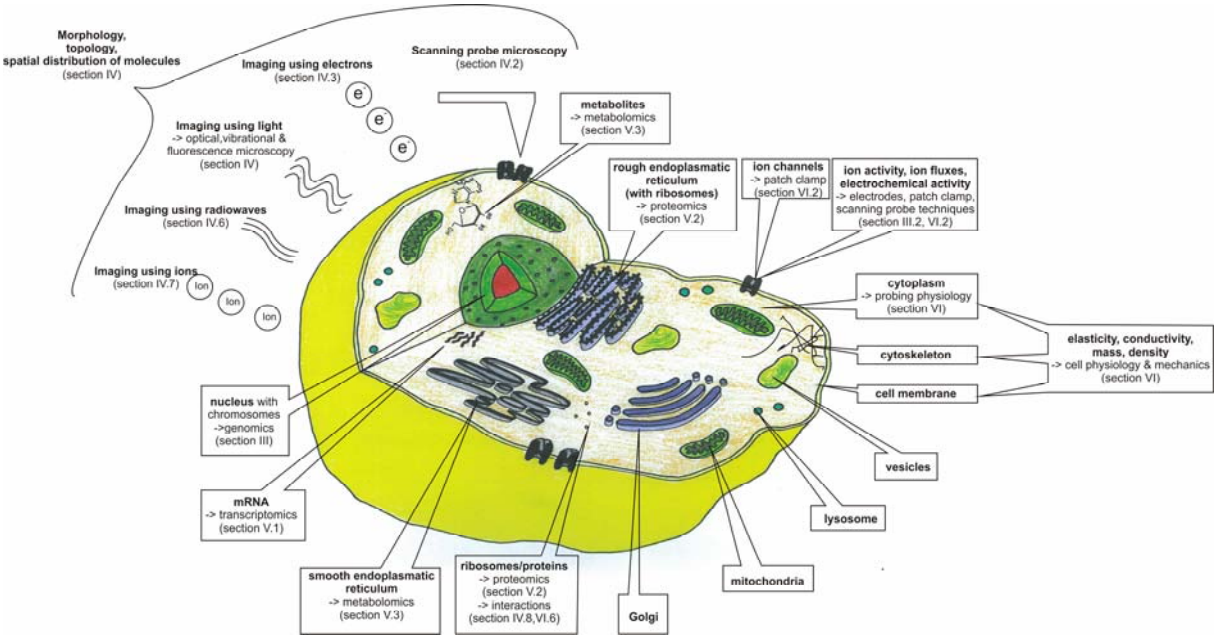


Figure 1*: Scheme of a single cell with its components (drawn not to scale) and an overview of the techniques that can be used to investigate and characterize the cell.*

II. Overview of technologies and methods

A single cell is a complex system which comprises of several different organelles and components. A schematic of a typical eukaryotic cell from human/animals is shown in Figure 1. When studying single cells a lot of different information from the cell is of interest, e.g., morphology, genotype, the molecular phenotype and various other physiological parameters and metabolic actions.

Morphological characteristics, “how the cell looks like”, can be assessed using a variety of different imaging methods. Many of those methods go way beyond just depicting size and

shape of the cell, but can reveal fine structure and even metabolic actions (section III). Imaging methods can be divided into label-free methods which use internal contrast of the sample and contrast-enhanced methods where a specific label, such as a fluorophore or radioactive label is introduced into the cell. Table 1 groups the different imaging techniques according to this criterion.

The oldest label-free technique is optical microscopy (as long as no dyes are involved to stain special organelles, see section III.1). Different types of scanning probe microscopy generate the image by scanning the surface of the cell with a small probe utilizing different sensing mechanisms (see section III.2). Electron (section III.3) and X-ray microscopy (section III.9) reveal the fine structure of cell organisation using electrons or X-rays. Different types of linear and non-linear vibrational spectroscopy (section III.4 and 5) make use of specific photon-matter interactions to generate information-rich images and mass spectrometry (section III.7) creates chemical specific images from the mass information of liberated ions from the sample.

The most prominent example for contrast-enhanced methods is fluorescence microscopy with its various special techniques (see section III.8). However, also in simple optical microscopy the contrast can be enhanced by using special dyes, which e.g. accumulate in the nucleus or other cellular organelles. Nuclear magnetic resonance imaging (section III.6) often utilizes paramagnetic metal particles to enhance contrast. Another criterion how to divide imaging techniques is whether they are destructive or non-destructive. This is especially important if several analysis techniques shall be combined to obtain a more complete picture of the cell of interest (see section III.10).

The widely established omic-technologies, i.e. genomics, transcriptomics, proteomics and metabolomics have tremendously advanced within the last years, making it possible to apply those techniques to the small sample volume contained in a single cell for deeper insight into structure, function, and dynamics on a single cell level (section III & V).

Other physiological parameters, such as ion concentration, mechanical or electrical properties are also essential for a proper function of cellular processes and correct interactions. A vast variety of techniques has been developed to probe those properties and will be discussed in section VI.

Ultimately, all the knowledge and insights gained from single cell analysis will find application in the study of more complex systems, such as tissues or full organs, where the individual cells act in their natural environment in close interaction and cooperation with all

the other cells of the same tissue or organ. A few examples of what is already possible now will be given in section VII.

Despite all the advances in single cell research there are still only a few examples of information gained being used in clinical diagnostics. However, it is expected that upon further progress in automation, throughput and general understanding of cellular heterogeneity, several interesting applications in different fields of medicine, such as immunology, regenerative medicine, cancer research or drug discovery, will emerge. (section X)

***Table 1**: Imaging methods grouped according to required contrast*

Label-free methods (internal contrast of the sample)	**Contrast-enhanced methods (specific label is applied)**
Optical microscopy (without stains)	Optical microscopy (with dyes to stain special organelles)
Scanning probe microscopy	Fluorescence microscopy
Electron microscopy	Nuclear magnetic resonance imaging with paramagnetic metal particles
X-ray microscopy	
Linear and non-linear vibrational spectroscopy	
Mass spectrometry	

III. Far more than morphology: Dynamic at the imaging front

Imaging techniques available for the analysis of single cells do not only yield morphological information and answer questions such as "What does the cell really look like?" and "What shape has the nucleus?" but also provide quantitative analysis of chemical species inside subcellular compartments, give molecular details of cellular processes and functions such as growth and death as well as interactions. Imaging techniques aim to detect, identify, visualize and track the spatial distribution of molecules. The interpretation of such changes in structure, organization and activity gives a deeper understanding of cellular processes. Imaging techniques can be divided into direct imaging techniques where intrinsic properties are probed without the need for labels (e.g. Raman spectroscopic imaging) and indirect imaging techniques which achieve higher contrast by the application of labels (e.g. fluorescence microscopy). Imaging probes can be photons, charged particles, such as electrons, atoms as well as sound waves.

In the recent years imaging techniques have been extended and improved to give better resolution, both in space and time. A variety of different methods is now available to study morphology and function, both qualitatively, but also quantitatively [1]. The combination of complementary imaging techniques can greatly amplify available information on structural features and allows the dynamic visualization of morphological changes. [2]

III.1 Optical microscopy – with and without contrast

The oldest method to obtain morphological information from a cell is to investigate it with incoherent white light. From the transmitted and reflected light portions a white light image can be obtained. Such an image depicts reality, however in a filtered, often reduced, and thus imprecise, changed way. It is necessary to be always aware of this limitation. In order to visualize a single cell magnification is needed. Consequently, imaging techniques usually rely on a microscope setup in some way.

Due to its simplicity the most abundant way to get an impression of a cell is to place the culture flask on an inverted microscope and have a look at it. Usually, the cell's shape will uncover immediately if there is a mesenchymal or an epithelial cell present. Often, also the nuclear shape is of interest, e.g. for the differentiation of the leukocyte subtype. Living cells are only thin optical objects and therefore, the contrast is not very high. This can be overcome by staining the sample prior to observation or by using optical contrast methods, such as interferometry (e.g. differential interference contrast (DIC) [3] or quantitative phase imaging. [4]

Phase measurements can be carried out in the full field mode, i.e. providing simultaneously information from the whole image field. This allows for spatial and temporal investigations with time resolution of a few milliseconds. Several label-free, multi-focus imaging methods rely on phase measurements, such as digital holographic microscopy (DHM), Fourier phase microscopy (FPM), diffraction phase microscopy (DPM), spatial light interference microscopy (SLIM), just to name a few. [4]

III.2 High resolution morphology: Electron microscopy (EM)

Transmission electron microscopy (TEM) has become a powerful tool for characterizing structures ranging in size from cells and viruses to small molecular complexes with almost atomic resolution in 3D and to link this information to the macroscopic properties. [5] Information on specifically bound elements like phosphorus, calcium and iron in compartments within sectioned cells can be gained by recording electron energy loss spectra (EELS) at each point of the sample. [6]

Vitrification can preserve biological cells in their near-native, hydrated environment. Automated low dose imaging is necessary to avoid irradiation damage. [7] Cryo-electron microscopy (Cryo-EM) is the method of choice for investigating radiation-sensitive specimens such as single cells. [8-13] In the process the biological sample is studied in a transmission electron microscope under cryogenic conditions. Macromolecular structures inside the cell can be revealed in 3D with a spatial resolution of 4 nm, providing an unprecedented insight into the cellular organization. [7] Cryo-EM can be further sub-divided into cryo-electron tomography, single-particle cryo-electron microscopy and electron crystallography.[5] Hybrid approaches of these techniques and X-ray crystallography as well as nuclear magnetic resonance (NMR) spectroscopy emerge and provide complementary information.

Further sample preparation techniques and instrument modifications for high sensitivity and good contrast as well as post-image processing procedures have been reviewed recently. [7]

In the publicly available repository Electron Microscopy Data Bank (EMDB; http://emdatabank.org/) 3D electron microscopic data of macromolecular complexes and cells can be found with a spatial resolution of 2 to 100 Å.

III.3 Scanning probe microscopy

There exists a vast variety of scanning probe microscopy techniques that all have in common that a physical probe (a kind of “tip”) is moved over the sample surface and records the

surface characteristics. Some of those techniques, such as atomic force microscopy, can achieve a very high spatial resolution. Others, such as electrochemical scanning probe techniques can provide information on mass transfer [14]. Four examples of scanning probe microscopy techniques are presented below.

III.3.1 Atomic force microscopy (AFM)

Atomic force microscopy (AFM) once developed as a high-resolution imaging tool of non-conducting surfaces has become a unique analysis method [15]. AFM overcomes the limited resolution of optical microscopy as well as the limited applicability of electron microscopy. The latter requires vacuum conditions and, therefore, is not appropriate for the investigation of biological samples in their native environments. In contrast, AFM can measure living cells directly in their culture medium without the need of cell fixation or staining. Imaging of cell surface morphology and membrane structure is possible as well as are investigations of dynamic processes involving molecules, organelles and other structures in living cells and interactions at the single-molecule and single cell level [16]. The measurements are based on physical interaction between a nanometer sized tip and the sample. The tip with its contact area of a few square nanometers is attached to the cantilever. This cantilever or the sample is moved in xy-direction via a piezoelectric scanner. A laser beam focused on the end of the cantilever is then reflected onto a photodiode detector. Due to the bending of the cantilever in response to the surface topography the detected laser beam moves and can be detected with pico Newton sensitivity. As a result, a topographic map of the surface is constructed. Generally, a resolution as high as 0.1 nm in lateral and 0.01 nm in vertical direction for molecular or even atomic imaging can be achieved [16]. Due to the fact, that unfixed mammalian cells are very large and soft, the resolution of AFM cell images is limited to approximately 50 nm [16]. Achieving a high spatial resolution down to the single-molecule level on living mammalian cells is still a challenge.

Improvements of the temporal resolution were made by the development of high-speed AFM. This nano-dynamic visualization technique is capable of observing structure dynamics and dynamic processes at a sub-second to sub-100 ms temporal resolution and a 2 nm lateral and a 0.1 nm vertical resolution[17]. The high-speed AFM imaging studies cover a wide range of dynamic molecular processes and structure dynamics, e.g. structure dynamics of proteins in action, self-assembly processes, dynamic protein–protein and protein–DNA interactions, diffusion processes, and molecular processes associated with enzymatic reactions [17].

To overcome the lack of biochemical specificity of the conventional AFM topographic imaging specific antibodies or ligands can be conjugated to the tip enabling the detection and

localization of single molecular recognition sites. Thereby, adhesion force mapping and dynamic recognition force mapping are possible. For details see the review by Hinterdorfer and colleagues [18].

Due to the open AFM setup, combinations with other tools for imaging and functional assays are possible. To select specific components in the heterogeneous cell for AFM characterization, AFM is usually combined with optical microscopy. Fluorescence microscopy is often used for investigations into clinical drugs. Combinations with several advanced optical spectroscopic methods gained impact, e.g. super-resolution imaging and enhanced Raman spectroscopy, allowing for real-time characterization of the molecular composition as well as structural changes. [16]

In the last years rapid progress has been made in AFM imaging of single cells to provide new information on cell surface structure, track the cellular dynamic process, evaluate drug activities and investigate mechanisms of drug action. For detailed information see the reviews by Shi and co-workers [16] and Dufrêne *et al*. [15].

Besides AFM imaging, AFM force mode has overcome some limitations and is capable of gaining quantitative information on cellular interactions at the single-molecule level. Studies concerning sample stiffness and viscoelasticity, cell adhesion, signal transduction and receptor mapping were made [19].

III.3.2 Scanning ion conductance microscopy (SICM)

Scanning ion conductance microscopy (SICM) is another scanning probe technique that can surmount the light diffraction limit and visualize the topography and local changes of living cells.[20] An electrolyted-filled nanopipette scans the sample and the ion current is measured. If the nanopipette is close to the cell surface, a non-conducting surface, the ion flow from the pipette opening is hindered. This relationship between resistance (ion current) and distance can be used to generate a three-dimensional topographical image of the sample surface. Different scanning modes have been developed which differ in speed and surface roughness that can be captured by this technique without destroying the probe [20]. The spatial resolution depends on the opening diameter of the nanopipette (resolution is roughly 3/2 of the diameter [21]) and values as low as 5 nm have been reported. Detailed three-dimensional SICM images enabled investigation of the morphological response of living cells on different stimuli, such as cytokines, corticosteroids, nanoparticles, shear stress or even surface changes after stimulated exocytosis. From the 3D image of the cell also information on the cell volume and volume changes can be gained. [20] The electrical distance control in SICM avoids mechanical damage of very soft and delicate samples such as living cells and unlike with AFM, it is

possible to image very fine and lose structures of cells, such as cell extensions, without any distortion. [22]

SICM can be combined with various other detection methods and act as a multifunctional tool: combination was reported with optical methods such as scanning near field techniques, fluorescence microscopy and even FRET; as well as with electrochemical recordings, such as patch clamp and ion-selective microelectrodes. Single-molecule fluorescence microscopy and SICM can reveal information on protein functions [20] as well as insights into cell membrane organization and function. [23] Combination of SICM and FRET can be used to elucidate pathophysiological mechanisms as was done to gain insights into the role of the redistribution of the β2-adrenergic receptor in heart failure [24] Combination with patch clamp with two glass pipettes can be used to generate functional maps of ion channels. [25] When the same pipette is used to first scan the cell surface with high topological resolution, and then for ion channel recording it is called "smart patch clamp" as the nanodomain for creating the seal with the surface can be chosen with high precision. [26]

The scanning tip can further be utilized to deposit molecules onto the surface or modify the local ion concentration. Such biochemical applications of SICM have been reviewed elsewhere. [27, 28]

III.3.3 Atom probe tomography (APT)

Atom probe tomography (APT) provides three-dimensional maps of ion compositions and was classically used to map metals and semiconductors with a sub-nanometer resolution. Recently, Narayan *et al*. were able to record cellular ions and metabolites from unstained, freeze-dried mammalian cells. Thereby, the reconstructions of cellular sub-volumes at high resolution revealed a surprising amount of spatial heterogeneity of specific chemical species within the cell [29]. APT is based on the field evaporation. Thereby, ions are desorbed from a needle-shaped sample by application of a very intense electrical field of several volts per nanometer under vacuum and at cryogenic temperatures, followed by the detection with a single-ion sensitive detector. The combination of APT with time-of-flight measurements allows the chemical identification of these detected ions in a mass spectrum.

III.3.4 Scanning electrochemical microscopy (SECM)

Scanning electrochemical microscopy (SECM) is suitable to monitor the electrochemical events on or in close proximity to a surface. Although, SECM cannot provide the same topographical resolution as atomic force microscopy or scanning electron microscopy, it is a

powerful analytical tool for the imaging of surface topography and the mapping of electrochemical activity of living cells on a sub-µm scale [30].

The key element of SECM is a small scaled electrode serving as a mobile probe and recording changes in electrochemical potential. This so-called ultramicroelectrode is characterized by its enormous sensitivity (enabling the detection of even trace amounts of analytes), its short response time and the high spatial resolution. It consists of an electroactive material (e.g., carbon, platinum, gold) and can have different shapes (e.g. disks, rings, bands, cylinders, spheres, hemispheres) depending on the application. Due to the electron transfer reactions of the detected analytes at well-defined potentials the electrochemical detection is highly specific. The small size of the electrode, which ranges from micrometers to nanometers, allows for an unhampered approach to cells and provides high lateral resolution for imaging [31].

Different imaging modes can be used, e.g. constant-height and constant-current mode. Constant-height mode imaging is the traditional scanning mode where the distance between the tip and the sample is stable and the tip scans across the surface in the xy plane. However, a feedback-based guidance system is required to maintain this distance. In the constant-current mode the device attempts to maintain a constant current by changing the substrate to tip distance. For more details see the review by Bergner *et al*. [30]

The use of ultramicroelectrodes to study single cells requires appropriate dimensions, stability during the whole experiment, high analyte sensitivity and selectivity and a high signal-to-noise ratio. Furthermore, biomolecules from the cell culture medium or even cells themselves can bind irreversibly to the electrode and consequently reduce the sensitivity. These requirements and handling problems still hold potentials for improvements.

SECM offers several advantages compared to other methods: (1) in contrast to fluorescence microscopy (section III.8) no staining or labelling is required and (2) unlike the AFM probe, the SECM probe does not need to touch the cell, thus time-lapse measurements without mechanically scratching the cell are possible [30, 32]. Nevertheless, most SECM imaging experiments were conducted with the addition of a certain redox mediator which is usually non-physiologic and undesired. However, Zhang and co-workers introduced dissolved oxygen as the redox mediator in the medium solution which provides an opportunity of label-free imaging of cellular morphology. [32] Quantifying the flux of molecules entering or leaving a cell, studying ion transport in channels, probing the local electrochemical reactions at and inside living cells are possible application fields of SECM. Furthermore, membrane permeability and the presence of metabolites can be detected and enzymatic activities can be

evaluated [31, 33]. Zhao and colleagues successfully detected reactive oxygen species (ROS) released from living macrophages [34] as well as single human bladder and kidney epithelial cells [35] by means of SECM. Real-time filming of the movement and morphological changes of living cells was performed via time-lapse SECM [32]. For a detailed insight into the application of SECM see the following reviews [30, 31, 33].

In addition, simultaneous investigations of (living) samples are possible by combining SECM with other biosensing techniques. Microfabrication techniques hold promise in supporting SECM-based investigation by providing fluidic-based culture platforms that can control cell environments at well-defined length scales. Integration of an inverted fluorescence microscope allows simultaneous or subsequent evaluation of the state of the cells following SECM measurements [31].

SECM in combination with optical microscopy is a powerful analytical tool to receive multidimensional information on complicated cellular processes. Therefor, integration into optical fibre probes, near-field optical microscopes, atomic force microscopes and confocal (laser) microscopes is carried out. The construction of a smaller optical fibre probe will enable electrochemical and optical imaging with nm-resolution [33].

III.4 Raman spectroscopic imaging

Raman spectroscopic imaging is a hyperspectral imaging technique that can provide information-rich chemical images in a non-invasive and non-destructive manner. Raw data of Raman spectral images include spatial xy-information as well as a spectral dimension which gives the vibrational signature of the overall molecular composition, e.g. from proteins, nucleic acids, lipids, carbohydrates and inorganic crystals. Addition of a spatial z-component is also possible. Spectra from different points of an image can be acquired in point, line or map mode depending on the applied optics and the capacity of the detector, which allows for different temporal resolution. The Raman effect is based on inelastic photon scattering of incoming monochromatic laser light on molecular vibrations. As almost all molecules are Raman active, no external label is required. However, since Raman scattering is a very rare event, detection of Raman spectra requires highly sensitive detectors. Although the technique had long been applied in chemical research, it was introduced only in 1990 for studies on single cells.[36] Raman spectra from mixed samples such as biological cells are very complex and require statistical analysis for meaningful interpretation. Up to now, Raman spectroscopy is an uncommon method in cell biology, but slowly entering the field. Improvements in detector technology [37] and data evaluation procedures led to some interesting Raman-based studies during the last decade which should further increase its relevance in single cell

research in future. Raman micro-spectroscopy could depict cell morphology and compartments such as nucleus, cytoplasm and vesicles.[38] Cytochrome dynamics have been studied during apoptosis.[39] Lipid droplet dynamics and composition were addressed in hepatocytes[40] as well as hepatic stellate cells[41]. With the help of Raman spectroscopy is possible to distinguish different cell types and build classification models which can assign the cell type based on the spectral characteristics [42-45]. For such single cells classification models, it is important to perform proper sample size planning and take random testing uncertainty into account. [46] Furthermore, Raman spectroscopy was used to identify pathological states of the cells, follow differentiation, transformation and viability as well as provide insights into metabolism and chemical reactions [42, 47, 48]. Also single cells in the tissue surrounding could be identified by means of Raman spectroscopy in combination with statistical analysis methods [49].

In combination with microfluidic Raman spectroscopy can be used to sort individual cells (**Raman activated cell sorting, RACS**). [50, 51] However, the achieved throughput is by far less than for fluorescence activated cell sorting (FACS) (see also section VIII.3).

One reason for that is the inherently weak Raman scattering efficiency. Several methods have been developed to increase Raman signal intensity. In **resonance Raman spectroscopy** the wavelength of the Raman excitation light matches with an electronic transition of the molecules of interests. The Raman bands of the vibrations that couple to that electronic transition experience an increase in intensity by 4-6 orders of magnitude. [50] Resonance Raman spectroscopy was used to study hemoproteins and monitor oxygenation state of red blood cells. [52] Furthermore, the technique was utilized to follow beta caroteine loaded nanoparticle uptake in living cells as possible drug carriers [53] as well as to follow differences in internalization via patch clamp between free haemin and peptide capped haemin into living HEK cells. [54]

Another method which can increase the observed Raman signal intensity is **surface-enhanced Raman spectroscopy (SERS)**. In the close vicinity to a rough metal surface (mostly gold and silver are used), an increase in intensity of up to 14 orders of magnitude is reported. [50] The used nanoparticles play a crucial role. [55] They can be functionalized and their uptake by single cells can be tracked.[56] Surface-enhanced Raman spectroscopy has been used to detect cancer cells and investigate treatment efficiency. [3]. Multiplexed optical sensing using SERS was reviewed by Rodriguez-Lorenzo *et al*. [57]

If the SERS-active particle is reduced to a single tip and combined with an AFM (see section III.2.1) one can speak of **tip-enhanced Raman spectroscopy (TERS)**. By means of TERS

the surfaces of single cells can be investigated with a high spatial resolution (down to 10-20 nm) [58]

The complementary vibrational spectroscopic imaging technique, **IR absorption spectroscopy**, uses light in the mid IR region to characterize the sample. The diffraction limited resolution is on the order of a few micrometers, the same size as single cells. [59] Therefore, IR spectroscopy is mainly used to study large cells, such as skin fibroblasts, giant sarcoma cells [60] and oral mucosa cells [61]. Using single spot measurements from individual cells it is possible to follow cell-cycle dependent variations [62] and conformational changes in DNA [63]. Furthermore, IR spectroscopy is successfully applied to identify single cells in tissue slices [64].

III.5 Nonlinear optical imaging

A thousand-fold improvement in the Raman signal intensity can be reached by generating **coherent anti-Stokes Raman scattering (CARS)**. Thereby, a target molecule is irradiated via two short-pulse laser beams (pump and Stokes beam). The frequencies of these beams must be tuned so that the frequency difference corresponds to a vibration of the target. In that case, coherently vibrating molecules in the sample volume will scatter the probe beam, resulting in a coherent signal [65, 66] . This multiphoton process offers intrinsic three-dimensional sub-µm resolution and an image contrast, which is obtained from inelastically scattered light by the vibrations of endogenous chemical bonds [67]

CARS is suitable to examine live cell dynamics with its high chemical specificity and its label-free and non-invasive character. However, the complexity and the high costs of the laser systems prevented a widespread application of CARS microscopy so far.

The major asset of CARS is the delivery of high signal intensity from lipid C-H bond stretches. Therefore, the majority of CARS applications involve lipid imaging and lipid quantification which have been real challenges in analytics before. This is because lipid-specific markers for fluorescence microscopy are difficult to produce and the labelling process often affects lipid localization and function. [67] Although, at first glance, the analytical focus of CARS microscopy on lipids may seem like a limitation, the ability to image them has provided valuable insight into a numerous diversity of biological processes in which lipids play an important role, e.g., in biological membranes, as energy storage molecules or as messengers in cell communication. [43, 65] Lipid vesicles inside HeLa cells, membranes of lysed erythrocyte cells, the growth of lipid droplets in live adipocyte cells, the organelle transport in

living cells as well as lipid storage in the nematode *Caenorhabditis elegans* have been readily visualized by CARS microscopy. The composition and packing of individual cellular lipid droplets has also been imaged using multiplex CARS microscopy. For more details see the review by Krafft *et al.* [43]

In addition, by tuning the CARS laser into amide I vibration, protein distributions in epithelial cells were visualized. Using the OH-stretching vibration of water, cellular hydrodynamics have been investigated with sub-second time resolution. The C-D stretching vibration also gives raise to a strong signal. Therefore, deuterium labels are used in many CARS experiments. [43] Furthermore, monitoring of dynamic cellular processes, such as lipid metabolism and storage, organelle movements, tracking of molecules within cells and imaging of exogenously added probes or drugs have been successfully accomplished. [65]

The CARS setup can be combined with other methods, e.g. microfluidic devices (CARS flow cytometry) [43] or two-photon fluorescence microscopy. These multimodal instruments pave the way for further applications. [67]

Stimulated Raman scattering (SRS) is another label-free imaging method, which overcomes some limitations of CARS microscopy. It is characterized by an energy difference between pump and Stokes photons, which is resonant to the vibrational frequency of a special chemical bond in the molecule. Following, non-linear interaction the excitation of that molecular vibration is stimulated. This is accompanied by energy transfer from the pump beam to the Stokes beam resulting in the intensity loss of the pump beam and the intensity gain of the Stokes beam. In case that the energy difference between the two laser beams does not match with the target molecule vibrational frequency, a non-resonant background appears. [68]

SRS allows imaging of biological molecules in living cells with a high resolution, sensitivity and speed. Lipid measurement, drug delivery monitoring and tumour cell detection are only some of the application fields of SRS. [68] This year Wei and colleagues demonstrated for the first time imaging of newly synthesized proteins in live mammalian cells with high spatial–temporal resolution via SRS combined with metabolic incorporation of deuterium-labelled amino acids. Importantly, this method can readily generate spatial maps of the quantitative ratio between new and total proteomes.[69]

Second harmonic imaging microscopy of biological specimens exploits a nonlinear optical effect known as **second harmonic generation (SHG)**, where the energy of the incident photons is scattered via a process of harmonic upconversion, instead of being absorbed by a molecule.[70] In detail, an intense laser beam passes through a polarisable material with a non-centrosymmetric molecular organization. A nonlinear mixing of the excitation light then

results in the generation of a wave at twice the optical frequency.[67] SHG strongly depends on the polarization state of the laser light and the orientation of the dipole moment in the molecules that interact with the light.[71] It is a label-free technique, which is typically used to detect collagen in the extracellular matrix. A major disadvantage of SHG microscopy is that the signal from cellular components is generally weak. However, second harmonic generation imaging can reveal the non-centrosymmetric and inhomogeneous structure of the object.[71] Reshak and co-authors detected strong SHG from the granal regions in the starch free chloroplasts of single cells. Upon illumination the chloroplasts changed their orientation, which affected the SHG signal.[71] So far, only a few studies of SHG on single eukaryotic cells exist.

III.6 Nuclear magnetic resonance imaging (NMRI)

Nuclear magnetic resonance imaging (NMRI) represents a further non-invasive imaging method. This technique is based on the molecular fingerprint resulting from the chemical shifts of resonance frequencies of nuclear spins within a strong magnetic field. [72] Therefore, NMRI is well suited to follow nanoparticles for cell localization in organs and tissue. Specific molecular probes that concentrate on target cells after their injection into living organisms can be localized via targeted NMRI, e.g., antibodies linked to ultra-small super-paramagnetic iron oxide nanoparticles that bind to tumour cells. [73, 74] The first NMR microimages of single cells were obtained from frog ova with a spatial resolutions in the order of magnitude of 10 μm [75] due to a combination of small-diameter radio frequency coils and high magnetic field. In 2000 Grant and colleagues demonstrated for the first time the feasibility of NMR spectroscopy to localize osmolytes and metabolites within single neural cells from the sea hare *Aplysia californica*[76]. Lee *et al.* acquired compartment-specific spectra of an oocyte from *Xenopus laevis* and monitored the uptake kinetics of an externally applied drug into the individual subcellular compartments *in vivo*[77]. Technical improvements, such as small-volume NMR probes, and an accompanying enhancement of the detection limit enables the investigation of samples with the sizes of typical eukaryotic cells. [73]

III.7 Mass spectrometry imaging (MSI)

Another label free method for successful chemical characterization is mass spectrometry imaging (MSI), which is well suited to image and profile individual cells and subcellular structures. Similar to other imaging techniques, e.g. Raman mapping (section III.4), one spectrum is acquired at each point of a spatially defined grid. In MSI this spectrum contains

mass information from that particular spot. Ion images showing the distribution of the selected peak can be generated by applying mass filters to the collected mass spectra.[2]

MSI offers several advantages compared to other imaging techniques: (1) it has a high chemical specificity; (2) no pre-selection or even knowledge of the analytes is necessary prior to the analysis and, (3) the number of co-registered ion images is, in theory, only limited by the number of distinct detected ions and the resolution of the spectra. [78] Nevertheless, mass spectrometry is an invasive method necessitating further advances in sample preparation and data analysis as well as sensitivity improvement for cell-scale experiments.

For an extensive review concerning mass spectrometry imaging of single cells see the one of Lanni and co-authors[78] or the one of Masyuko *et al*.[2]

To answer the broad range of analytical as well as biological and biomedical questions secondary ion mass spectrometry (SIMS) and matrix assisted laser desorption/ionization mass spectrometry (MALDI MS) are the two most established mass spectrometry techniques to obtain morphology related chemical information from single cells.[79] SIMS is a surface analysis method providing chemical information from the first few nanometers of the sample surface. SIMS can routinely reach the highest spatial resolution at the sub-micron level (< 50 nm) in comparison to other MSI techniques. In addition, it delivers useful quantitative information. [80] Thereby, the detectable ions are limited to a small mass range of a few hundred Dalton. With this technique it is possible to localize analytes in two- as well as three dimensions within single cells. [78]

SIMS can be divided into dynamic and static SIMS. Dynamic SIMS is usually combined with other high-resolution imaging techniques, e.g. electron-, atomic force- and fluorescence microscopy.[78] High-resolution mapping of endogenous and exogenous ions and molecules provides unique insights into single cells: the localization of proteins and nucleic acids by detecting elemental sulphur and phosphorus is possible[81] as well as the localization of iron in diseased cells (which found applications in Alzheimer research) [82, 83]. Due to the detection of endogenous inorganic ions, e.g., Na^+ and Cl^-, information on the physiological state of the cell including mitosis, membrane potential and transport can be derived. [84] Furthermore, localization[85] and evaluation[86] of the efficiency of pharmaceuticals as well as direct cancer detection[87] are possible. For detailed insights into the applications and capabilities of dynamic SIMS the interested reader is referred to the article by Chandra in "The Encyclopedia of Mass Spectrometry" [84].

Static SIMS (also known as time-of-flight (TOF) - SIMS) is conveniently combined with a TOF analyzer acquiring full mass spectra for each pixel. Static SIMS is characterized by an

undamaged surface after measurement due to a limitation of the primary ion fluence of less than 10^{13} ions per cm^2. Due to this static limit, the application to cellular imaging is confined to membrane-localized molecules including membrane phospholipids[88] and other small molecules, e.g. cholesterol and vitamin E[78].

One of the most important efforts was achieved by Steinhauser and colleagues, who developed multi-isotope imaging mass spectrometry. This SIMS-based technique applied to individual cells from diverse organisms (Drosophila, mice, humans) allowed the measurement of stable isotope-containing functional tracers. Subcellular spatial resolution and quantitative information are among to the methodological achievements of this work. [89]

Matrix assisted laser desorption ionization (MALDI) MS is well-established for tissue-based studies, but has become routine for subcellular investigations. Usually, ultraviolet lasers are used for laser desorption ionization. [2] MALDI MS is the most versatile and easy-to-use MSI technology in order to identify the molecular signature of pathological phenomena [90]. This technique offers a large mass range, a high sensitivity for detection of analytes and is also suitable to investigate complex mixtures. Thereby, the sample preparation is easy, although the lack of appropriate matrix application methods, which limits the resolution of imaging, leaves room for improvements[91-94]. For MALDI MS measurements the sample is embedded in a matrix of organic substance crystals or liquid crystals and scanned by a focused, pulsed or continuous laser beam. The analyte molecules are (1) desorbed due to the energy absorption by the matrix, (2) ionized, (3) extracted from the source, (4) analyzed by their mass-to-charge ratio, and finally (5) detected. Thereby, a localization of metabolites, proteins, peptides and lipids as well as DNA and RNA is possible. MALDI MSI was successfully applied in single cell and organelle profiling studies (reviewed by Lanni *et al.*[78]). Improvement of the spatial resolution for cellular and sub-cellular investigations was one the main challenges in the last few years. Optimizations in the instrumental setup of MALDI MSI achieved a routine working resolution between 4 and 7 µm. [95-98] Spengler and Hubert were able to reach an imaging resolution between 0.6 and 1.5 µm via SMALDI (Scanning microprobe MALDI).[99] The capability of SMALDI was shown by imaging human renal carcinoma cells with a resolution of 2 µm and detecting masses up to 5 kDa[100]. Combination of MALDI MSI with other methods, e.g., Raman spectroscopic imaging, infrared imaging or Fourier transform ion cyclotron resonance MS, would truly benefit MALDI MSI informative value [90]. Also, combination of other MS-based techniques, e.g., matrix-free laser desorption/ionization MS, with fluorescence and Raman microspectroscopy gains relevance, as it was shown by a multidimensional chemical analysis of *Euglena gracilis* and *Chlamydomonas reinhardtii*.[101]

Furthermore, three-dimensional cross-sectional images of rat brain and plant tissues could be constructed by laser ablation electrospray ionization (LAESI) MS combining two-dimensional lateral imaging with depth profiling. Shrestha *et al*. demonstrated in situ cell-by-cell imaging of plant tissues. Chemical imaging of the metabolite cyaniding (purple pigmentation in onion epidermal cells) using single cells as voxels reflects the spatial distribution of biochemical differences within a tissue.[102]

In addition, scanning near-field optical microscopy (SNOM) MS, laser ablation-inductively coupled plasma (LA-ICP) MS and nanostructure-initiator mass spectrometry (NIMS) have a high potential as future methods for subcellular investigations since they work under atmospheric conditions and are therefore capable to image living cells[78].

III.8 Fluorescence microscopic imaging

Fluorescence microscopic imaging is a commonly used approach to visualize cells and organelles, and to study intracellular interactions. A wide range of organic fluorophores and fluorescent proteins is available that can be selectively inserted into a cell or even expressed therein. During the last years also luminescent heavy metal complexes [103] and quantum dots [104] were made biocompatible to be used in live cell imaging. Quantum dots have the advantages of being chemical and photo-resistant reporters with a narrow and tuneable emission ranging from UV to NIR. [104] They could even be used for single biomolecule tracking inside living cells. [105]

Two main challenges are faced in the field of fluorescence-based microscopy. On the one hand high spatial resolution is of importance if nearby molecules inside or on top of the cell shall be separated. On the other hand temporal resolution is limiting if dynamic events in living cells are of interest. An essay emphasizing critical aspects for live cell imaging was published recently by Sung[106].

III.8.1 Confocal Laser Scanning Microscopy (CLSM)

To achieve spatial resolution in z-direction confocal laser scanning microscopes either equipped with a single pinhole or a scanning disk for faster data acquisition are still widely used. The principle of confocality is based on the conjugation of the sample plane, where incoming light from the objective is focused and a pinhole in its optical plane, where emitted light from the sample plane arrives. The review by Stehbens *et al*. [107] describes recent developments of confocal imaging setups.

An intriguing recent application is the detailed observation of lamina associated domains (LADs), the missing of their heritability and their spread after mitosis. LADs are sites of

contact between DNA and nuclear lamina. Imaging is based on enzymatic adenine-6-methylation in DNA in case of its contact with the lamina and subsequent fluorescence-labelling by the fragment of another enzyme. The modifications work as an event memory. Both active proteins are only expressed after transfection. While the authors state randomness of LAD establishment, the study also points to possible influences of LADs in gene regulation.[108] This represents another hint for the plasticity the cell type concept has to fulfill. Others concentrated e. g. on the elucidation of the moment of nuclear pore formation during reestablishment of the nuclear envelope. Evidence was found that all components join in the nascent envelope and therefore, against the assembly or maintenance of a prepore complex at endoplasmatic reticulum membranes.[109]

Also in the field of cytoskeletal research confocal LSM still reveals valuable information. Dunsch and collegues e.g. reported on spindle orientation during mitosis depending on a special dynein light chain[110].

III.8.2 Revealing of molecular interactions: FRET and FLIM

Fluorescence or **Förster resonance energy transfer (FRET)** experiments have been applied to study inter- or intramolecular interactions since decades [111, 112]. For a recent review read e.g., the one by Zadran *et al.*[113]. FRET exploits a radiationless process transferring energy collected via excitation from an electronic ground state to the first excited state by a first fluorophore, called the donor, to a second fluorophore, called the acceptor, which emits a photon while relaxing back to its electronic ground state. The photon emitted by the acceptor is red shifted compared to the photon that would have been emitted by the donor without the occurrence of FRET. Donor and acceptor constitute a FRET pair. There are several requirements to be fulfilled for FRET to happen. First of all, the emission spectrum of FRET-donor and the excitation spectrum of FRET-acceptor have to overlap. There are several FRET pairs commercially available. For a list of widely used fluorescent proteins, represented also in FRET pairs see Wiedenmann *et al.*[114]. In addition, the orientation of the transition dipole moments of the FRET pair influences FRET efficiency. Illustrative DFT calculations concerning several FRET pairs where published by Ansbacher and colleagues[115]. Finally, the characteristic making FRET a valuable tool for interaction studies is its distance sensitivity. Since the efficiency of FRET decreases proportional to the sixth power of the distance between donor and acceptor, FRET is known to occur only within a molecule distance of 1 to 10 nm. Nowadays, FRET is applied to whole cells to study e.g. HER2-EGFR dimerization[116] or spatio-temporal kinase activity[117]. Cyan fluorescent protein (CFP) – yellow fluorescent

protein (YFP) FRET tools were presented furthermore, to sense cellular energy state via adenosine triphosphate (ATP) recognition.[118, 119] Notably, ATP action as an energy transition quencher is disputed by a more recent work assigning the ATP effect rather to interaction of ATP and the FRET-donor.[120] The adaptability of FRET experiments is still limited due to strict spectral requirements for fluorophore combinations challenging the synchronous detection of parallel events in the cell. Methods like compartmentalization or the coupling of one donor to different acceptors to overcome these confinements are discussed in a recent review by Deprey *et al*.[121]. Geißler and co-workers even described the possibility to distinguish acceptor emissions of five different FRET acceptors in one experiment by employment of a single donor (Tb complex) for energy transfer and advanced spectral crosstalk correction, although not in single cells but in serum.[122] Ouyang and colleagues were able to study Src kinase and MT1-MMP activation simultaneously upon stimulation in living HeLa cells using two completely different FRET pairs.[117]

FRET enables not only the detection of the fluorescence of an acceptor fluorophore that has not excited by the incident laser beam but also shortens the time the donor is in the excited state. This results in a decrease of its fluorescence life time. Thus, interaction can also be concluded from changes in donor fluorescence life time when an acceptor is e.g. coupled to an enzyme substrate or a receptor ligand. Studies of intra-molecular changes in living cells are also possible. As an example, the epidermal growth factor receptor conformations in free and ligand-bound form were examined only recently to address its ability of self-inhibition.[123]

The fluorescence lifetime is sensitive to changes in the molecular environment. Thus, information on ion concentrations, pH-values or oxygen presence can be derived from a change in fluorescence lifetime. The possibility to distinguish several fractions of a certain fluorophore inside one cell makes **fluorescene lifetime imaging microscopy (FLIM)** especially advantageous. [124] For a detailed description of FLIM and further exemplary applications in living cells see Sun *et al*.[125]. For an overview of different FLIM techniques and their microscope implementation see Becker. [124]

III.8.3 Following motion inside the cell: FRAP and FCS

Fluorescence recovery after photobleaching (FRAP) allows time resolved observation of molecule dynamics. It requires the molecule of interest to be tagged or fused to a fluorophore and the photobleaching with intense (laser) light of a region of interest. After the bleaching event, reoccurrence of fluorescence in that area is detected. Furthermore, it is possible to flip the experiment around by bleaching an area adjacent to the observed field and to follow

fluorescence loss postbleaching. For a review covering the fundamentals of FRAP see the one by Reits and Neefjes[126]. For a critical, more recent review with focus on nuclear protein dynamics the one by Mueller *et al.*[127] is recommended. Recently, FRAP e.g. served to visualize H-Ras exchange in free diffusion between B- and T-lymphocytes via membranes of tunnelling nanotubes[128] or heat shock factor 1 dynamics[129]. Advancement in measurement precision by combining FRAP and **fluorescence correlation spectroscopy (FCS)** was described by Im and colleagues[130]. Both techniques complement each other with respect to sensitivity and speed of the detectable reaction kinetics.[129, 130]

In brief, for FCS a defined volume, the focal volume, is laser illuminated and the resulting fluorescence is detected continuously. Fluctuations in the fluorescence read out over time allow for the calculation of the diffusion coefficient of the fluorescing particle. Binding events and dissociation constants can be concluded from FCS measurements, in addition. For an exhaustive description of the FCS principle the review of Elson is recommended [131]. FCS is beneficial for single cell application, as is demonstrated by a number of FCS-based studies on molecule mobility, e.g. on nuclear receptor DNA binding[132] or signal transducer activity in cellular stress response[129] as well as on apoptosis onset based on caspase activity assessment[133, 134]. A dual-colour version of FCS is **fluorescence cross-correlation spectroscopy (FCCS)**. For FCCS measurements two spectrally distinct fluorophores are required. Diffusion dynamics are measured for each of the fluorophores as in conservative FCS. However, the results are cross-correlated afterwards, thus, revealing simultaneous or independent movements. The fluorophores can ,e.g., be situated at two members of a protein complex or both on one target molecule of an enzyme on the different sides of the restriction site. Transcription factor association[135] or enzyme activities[136, 137] were studied that way. For a review on FCCS, dealing also with further examples, see the one by Bacia *et al.*[138]

III.8.4 Super-resolution fluorescence microscopy

Confocal laser scanning microscopy is limited in lateral and axial resolution by diffraction. However, quite often it is of interest to observe also smaller structural detail and features in a cell. Several microscopy techniques evolved to gain resolution beyond the diffraction limit. Stimulated emission depletion (STED), structured illumination microscopy (SIM), photo-activated localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) are famous representatives. For a generalized application oriented review the ones by Ball *et al.*[139] and Schermelleh *et al.* [140] are suggested.

Spatially addressed modulation:

Stimulated emission depletion (STED)

In STED de-excitation of fluorophores is exploited by de-exciting a ring-shaped area using stimulated emission and detecting remaining spontaneous fluorescence in the avoided central space afterwards. A lateral resolution down to 50 nm was reported by Jans *et al*. [141]. However, due to point by point scanning and fluorescence detection the acquisition of a STED image is time consuming.[142] The panel of applicable fluorophores is limited as well.[142] A related methodology exploits reversible saturable optically linear fluorescence transitions (RESOLFT) using special photo-switchable fluorophores which can be kept in the non-fluorescent state for prolonged time, thus allowing for lower laser intensity for the on to off-switch. [3]. STED was applied to study neuronal architecture events on synapses.[143, 144] Distribution of TOM20, a component of the translocase of mitochondrial outer membrane (TOM) complex, was addressed by Wurm and colleagues. They found it clustered and in varying quantity depending on cell position in a colony.[145] In a recent contribution by Jans and co-workers the resolution capacity of STED served to study the organization of MINOS complexes in mitochondria. The authors report on highly ordered mitofilin, MINOS1 and $CHCHD_3$ structures.[141] TOM20 and MINOS abundance differences with respect to mitochondrial nuclear distance were uncovered in either study.[141, 145] For a review concerning STED and further high resolution microscopy techniques see Tønnesen & Nägerl[146] or Lidtke & Lidtke[147].

Structured illumination microscopy (SIM)

Another approach to improve the spatial resolution in light microscopy is pursued by SIM. For this full-field fluorescence method structured excitation light, exhibiting spatially varying intensity is used instead of homogenous illumination. Multiplicative meeting of incoming fine structured light with the fine structural details of the sample leads to specific patterns of emitted fluorescence, called Moiré fringes. Back calculation of the originally light emitting sources, meaning sample structure, from the measured data is possible as far as enough images under different illumination conditions are available. The required number of images depends on the pattern of the excitation beam. Each image has to be acquired with a different phase of the excitation light.[148] A two fold increase in resolution compared to wide-field imaging was simultaneously achieved in lateral and axial dimension.[140, 148] A resolution of 50 nm in a fluorescent bead sample has been shown for saturated SIM which involves a non-linear modulation pattern.[149, 150] For a review of super-resolution microscopy methods

containing especially informed knowledge on SIM see the one by Schermelleh, Heintzmann and Leonhardt.[140] 3D-SIM led to unprecedented accuracy in replication foci quantification, revealing a three to five fold higher number than CLSM-based counting and a decrease in amount during cell cycle S phase.[151] Moreover, stratification between space occupied by nuclear pore complexes, nuclear lamina and chromatin was visualized impressively, pointing furthermore to a strict separation of nuclear pores and chromatin.[152] In addition, perfect matching of 3D-SIM and fluorescence in situ hybridization has been shown by Markaki and co-workers who report on diverse examples concerning the spatial extend of heterochromatin interaction in interphase.[153]

Stochastic modulation: Point-localization super-resolution microscopy

The term point-localization super-resolution microscopy or pointillism – as suggested by Lidke *et al*. [154]- comprises amongst others PALM and STORM. The techniques share the strategy of repeated stochastic fluorophore activation followed by wide field image acquisition and calculation of exact fluorophore positions from a set of such images acquired from the same field but with stochastically varying fluorophores in the "on" state. These calculations are based on precise fits of detected single molecule emissions. As a review focusing on point-localization super resolution microscopy strategies and their background that also contains application examples the one of Sengupta *et al*. [155] is recommended.

A derivative method introduced by Dertinger *et al*.[156] is super-resolution optical fluctuation imaging (SOFI) which relies on flickering fluorophores. For SOFI a number of images have to be acquired as well. In SOFI higher-order fluctuation statistics is exploited without the need to localize individual molecules. A simple variance projection is an example of a SOFI image of order two. SOFI was already shown to improve resolution and contrast and to suppress background in living HeLa cells also in a two-colour version.[157] SOFI typically works with relatively dense concentrations of simultaneous emitters, but yields only moderate resolution (100nm typically). Accepting the confinement of several possible solutions for molecular assignments in dense data led to the development of 3B (Bayesian analysis of blinking and bleaching) which enables live cell imaging of podosomes at 0.5 Hertz with a resolution of around 40nm [158]. Using the example of those small podosomes (diameter 0.5-2µm), which sit like small "adhesive bowls" on the outer membrane of macrophages, Figure 2 illustrates the spatial resolution power of a few of the introduced high resolution approaches compared to widefield microscopy and confocal laser scanning microscopy.

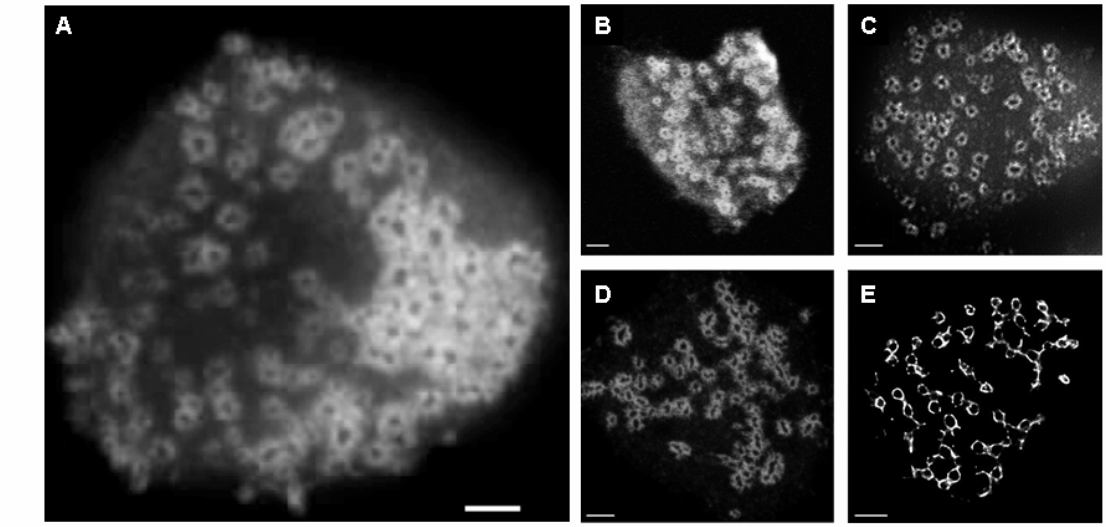


Figure 2*: Illustration of the spatial resolution of the different microscopy techniques using the example of the podosome ring of actin associated proteins in THP-1 cells (macrophages). The membrane-cytoskeletal protein vinculin was labelled with Alexa488 (A-C, E) or ATTO-647 (D) for visualization. All scale bars are 2 µm. (Figure adapted from Walde et al.* [419] *and Cox et al* [158]*).*
A: *Widefield image of a macrophage. The cylindrical actin-rich podosomes (0.5-2µm in diameter) can be seen as bright circles against a blur background. (section III.8)* ***B****: Confocal image, as the podosomes are very thin structures with only minimal depth profile (they form a flat sample with all the podosomes close to the cover slide surface) the sectioning difference between widefield (A) and confocal (B) is relatively small. (section III.8.1)* ***C****: SIM image. (section III.8.4)* ***D****: STED image recorded on a commercial instrument. Even better resolution can be achieved with home-build STED systems. (section III.8.4)* ***E****: 3B image. No background fluorescence interferes with the signal from the podosomes. (section III.8.4)*

III.8.5 Hyperspectral fluorescence imaging

While the techniques described above are suitable to achieve a spatial resolution down to 50 nm (lateral resolution achieved by STED)[141], they suffer from time consuming pointwise fluorescence detection (CLSM, STED) and the necessity of repeated image acquisition (SIM, PALM, STORM, SOFI), respectively. If different fluorophores are of relevance, acquisition time raises accordingly and in addition, due to the necessity of mechanical configuration switches. Acquisition of hyperspectral image maps overcomes this problem. Hyperspectral means the detection of spectral regions covering emission ranges of different fluorophores on a CCD (charge coupled device) at ones instead of a narrow part to avoid overlap of emission. Information about the presence of a certain fluorophore at a point has to be derived from

deconvolution afterwards. Uhr and co-workers employed a hyperspectral imaging device for quantitative assessment of 10 independent markers in individual breast tumour cells from solid tumour tissue as well as circulating tumour cells. A tumour molecular signature for solid tumour is stated.[159] An image mapping spectrometer for fluorescence measurements avoiding the scanning obligation was described by Gao and colleagues[160]. It enables to acquire maps with 285 x 285 pixels at a time rate of 7,5 frames per second (fps). Simultaneously, the full spectral information (60 channels with an average sampling interval of 3.3 nm) is projected from each sampled pixel position onto a large format CCD detector. Thus, data acquisition at a single moment in time results in a 3D matrix (data cube) containing x- y- and λ-information.[160] In a recent contribution Elliott and colleagues report the use of an adapted version to study intracellular cAMP and Ca^{2+} concentration dynamics simultaneously in living representatives of a pancreatic β-cell line at 2 fps upon glucose stimulation. They reveal anti-correlation of the concentration oscillations of these signal transducers.[161] As an additional advantage, Leavesley and colleagues state the superiority of hyperspectral fluorescence detection compared to the narrow band mode with respect to sensitivity and specificity of GFP positivity determination of single cells against highly fluorescent background in pulmonary tissue.[162]

III.8.6 Total Internal Reflection Fluorescence Microscopy (TIRFM)

A possibility to study the distribution of molecules in- and outside the cell body is total internal reflection fluorescence microscopy (TIRFM). It is based on light refraction at the interface of media with unequal optical density. If the incident beam passes from the medium with higher refractive index to the one with lower refractive index, there is a certain critical angle depending on these refractive indices from which any light can pass [3]. Nevertheless, a non-propagating electromagnetic field exists, the evanescent wave, which excites fluorophores close to the phase border. Since the field strength decreases rapidly with the distance to this border excitation within the field is highly selective avoiding out of plane fluorescence reliably.[3, 163] Several of the afore described super-resolution microscopy techniques are applied preferentially in TIRFM mode because of the accompanying improved signal to noise ratio. [3, 157] Due to the same effect TIRFM is restricted to studies of cell surface molecules as E-cadherin[164] or intracellular events taking place close to[165, 166] or at the cell membrane like vesicle fusion on the other hand. Synaptosomal-associated protein (SNAP) dynamics involved in exocytosis for instance where addressed by Wang *et al*. in an approach combining FRET and TIRF.[167] For further insights and recent instrumental advances as well

as more application examples see the section dedicated to TIRFM in the comprehensive review by Stender *et al*.[3]

III.9 Imaging techniques just around the corner: SPRI, XRM, XAS, XRF

Despite of the impressing insights super-resolution fluorescence microscopy, electron microscopy and the other previously described advanced imaging techniques revealed up to now, each of them suffers from its own limitations. A step further or deeper always promises interesting new aspects – there is an unlimited demand for improvements and new developments. That is why two not that established single cell imaging techniques shall be shortly presented here.

A method promising to observe single cell reactions in real time and to be furthermore applicable in medical diagnosis is **surface plasmon resonance imaging** (**SPRI**). The surface plasmon resonance effect, the attenuation of light reflection at a phase border, results from resonance of an evanescent wave with a metal plasmonic field. A simple SPR setup might consist of a prism with a gold film on top. The evanescent wave develops if light passes from a medium with higher refractive index at an angle above a critical angle to a medium with lower refractive index. The plasmon is derived from electron gas movements in the metal layer. SPR happens at a specific angle of incoming light beam and phase border, the resonance angle. This resonance angle is highly sensitive to changes at the surface disturbing the plasmon which was exploited for long time in binding assays. [168] Capturing of cells based on surface molecule expression was described. [169] Recently the capability of SPR measurements to sense variations in single cell refractive index was reported and different SPRI devices were described [170, 171]. A review concerning the technique and its potential for allergy tests is available from Yanase *et al*. [168]

Insights into the presence, distribution and oxidation state of trace metals can be derived from **X-ray microscopy** (**XRM**), thus **X-ray absorption measurements** (**XAS**) and **X-ray fluorescence** (**XRF**). [172] Due to the availability of synchrotron radiation sources and improvements in X-ray optics, it is now possible to address subcellular compartments. XAS is based on the ejection of an electron from an atom shell at a certain energy amount which is reflected in the absorption spectrum. To enforce such an event sufficiently high energies are needed. The resulting electron hole is refilled by an outer shell electron which leads to element characteristic X-ray fluorescence (XRF) in addition. XAS can be operated in transmission or fluorescence mode. [173, 174] XAS images providing 50 nm lateral resolution and visualizing several organelles without the addition of contrasting chemicals have been

published. [175] Further advances might be associated with the inset of nanoparticles [176] and lensless setups [177]. XRM convinces with respect to resolution, intrinsic contrast and tomographic capabilities. [178] However, X-rays will always be harmful for live. Thus, prolonged live cell studies stay unfavourable.

III.10 Multimodal imaging: correlating results from different approaches

Electron and X-ray microscopy allow high spatial resolution and excellent intrinsic contrast; however, sample cells do not survive these procedures. Raman spectroscopy and MSI datasets brim over from chemical information; but it is necessary to assign biological relevance to the detected changes in chemical composition. The diverse fluorescence based microscopy techniques deliver colourful images; however, they rely on artificial labelling and are blind for non-labelled subcellular morphologies. The question arises, if the image they depict is the truth?

Apart from the development of new and advanced methods in order to visualize finest structures and detect minimal molecule traces in front of overwhelming noise multimodal imaging approaches prosper. Multimodal imaging is the combination of at least two imaging techniques usually in a sequential manner. [2] That way, information derived from the cell is amplified at the one hand and integrated at the other one. Multimodal imaging faces its own complications. First of all, the desired techniques need to be compatible with respect to sample preparation requirements. Anyway, this will be often achieved by a reasonable technique flow. A fluorescence label will interfere with Raman measurements but a Raman measured sample is still suited for fluorescence staining. In general, the more invasive or even destroying method has to be applied as the final one. In contrast, the assignment of corresponding datasets derived from different imaging approaches is inevitable. The development of automated registration algorithms is challenging due to varying magnifications, fields of view and underlying physics for the differing imaging techniques. [2, 179] Nevertheless, successful correlation of live cell confocal fluorescence imaging and SEM is described in a recent publication by Murphy *et al*. [179] Their multimodal approach alleviates feature identification in large data amount SEM stacks by registration of SEM data with fluorescing particles. Compatibility of Raman spectroscopic imaging and MSI was described by Li *et al*. [180] A recent review on correlative imaging focusing on the complementarities of the latter techniques is available by Masyuko *et al*. [2]

IV. Mapping the genome - tracing back the cell origin

The advent of DNA sequencing methods to identify the order of presence of the four different bases in DNA molecules opened the possibility to unfold the whole genome sequence of an organism. This facilitates the study of evolutionary relationships, genetic diseases and variations, and functional assignments of genes based on predicting their confirmation from their amino acid sequence. Genome sequencing of single cells only arose few years ago with the availability of less expensive and high throughput next generation sequencing techniques. Although mainly used to determine the diversity of non-culturable microorganisms in environmental samples [181], single cell genotyping can identify abnormal, mutated cells in a tissue or organism, which has a great potential for some diagnostic applications, especially to identify cancer cells or genetic defects in oocytes and sperms used for *in vitro* fertilization.

The main difference of single cell genome sequencing in comparison to sequencing a cell population is that the cells need to be isolated and that the genome needs to be amplified to obtain enough DNA for the sequencing process. This is usually done by multiple displacement amplification (MDA) that uses the DNA polymerase from phage Phi29 to copy the DNA with high fidelity[182] .

New technologies, such as combination of sequencing with microfluidics, allow sequencing at a subcellular scale, for example by separating two homologous copies of one chromosome from a cell in the metaphase, in order to determine different allele variations and meiotic recombinations[183]. For cancer diagnostics based on single cell genotyping, genome copy number quantification can reveal clonal subpopulations[184] , and sequencing of certain genes known to be often mutated in a special cancer type, can help to advance cancer diagnosis based on single cells[185] . In this regard exome sequencing is especially useful, as it allows studying more cells in a shorter time due to sequencing of only the protein-coding exon regions of a gene. This makes it a convenient tool to study cancer development and the tumour biology in special cancer types in order to find common biomarkers and to identify frequently and less frequently occurring mutations[186, 187].

V An even deeper look at the molecular phenotype of a cell

Classically, the phenotype is known to comprise all the visible values of attributes of an individual in contrast or reflection of its genotype (see section III), where these attributes are coded. Visible characteristics of cells enable to separate them into groups, as epithelial or mesenchymal or, with more specificity, e.g. as glial or neuronal. More pronounced

discrimination by presence or absence of certain molecules e.g. multi-drug resistance transporters, is also common and is especially important in pathology. However, the more molecular analysis techniques advance and the higher the throughput, the more attractive a separation according to the molecular phenotype becomes. The molecular phenotype, for the purpose of this review, is regarded as the sum of information concerning the presence of specific molecules (e.g. proteins, mRNA) as well as its overall chemical composition available for an individual cell.

Differential expression of genes gives rise to the diversity of cellular phenotypes. The presence or absence of their expression products - mRNA and proteins – is of special importance to characterize the molecular phenotype. With respect to mRNA studies, polymerase chain reaction (PCR), a technique well known from multi-cell analysis, has been adapted to be performed on single cell level. Advances in RNA sequencing and fluorescence in situ hybridization (FISH), however, make these approaches serious concurrence technologies. Improvements in spatial and temporal resolution in fluorescence detection systems have been achieved for RNA FISH and for the analyses on protein level, enabling new insights into cell cycle, signalling, behaviour and further more. Accordingly, proteomic and metabolomic information become more and more accessible even though, they are hard to assess on single cell level because their constituents cannot be amplified as easily as nucleic acids.[188]

A broader aspect, the overall chemical composition, is covered in the advancing field of biophotonics (section III). Spectroscopic data contain a spectral fingerprint of the cell, which also takes into account further cellular components as lipids in particular. In addition imaging of larger scenes with single cell recognition is possible.

V.1 Single cell gene expression analysis

The first step from genome to phenotype occurs at the level of transcription by copying the genetic information into a transportable messenger ribonucleic acid (mRNA) molecule. This mRNA then exits the nucleus and enters the translationary machinery of ribosomes in the cytoplasm in order to be used as a template for protein synthesis. This process is known as translation. Altogether, this gene expression process is pretty complex and the object of regulation at different levels and at several time points in various cell types underlining the importance to study gene expression on the single cell level.

There is a number of reviews concentrating on gene expression analysis at the single cell level, e.g., the ones by Tischler and Surani [189] or Stahlberg and Bengtsson [190] are recommended.

Multicellular organisms and tissues of higher animals are composed of many different cell types with the same genetic information but highly specialised in function. Thus, all these cells differ more or less in phenotype, making it necessary to study them on a single cell level to assess their function in the tissue and to understand the effects of stimuli from other cells (of same and different) types in the surrounding tissue. However, there are substantial cell-to-cell variations in gene expression even in cell populations of unicellular organisms with the same genotype.[191] These variations have been first observed in bacteria, but later also in eukaryotic cells. The variations can be described as the result of (1) extrinsic stochasticity, which is variation due to different activities of different regulatory molecules, and (2) intrinsic stochasticity, which is noise from gene expression itself due to random effects.[192] Stochasticity has been analysed in eukaryotic cells as well, and this revealed that gene expression frequencies and intensities can differ a lot between different eukaryotic genes. [191, 193, 194] Thus, single gene expression analysis is of utmost importance to identify gene regulatory networks by studying which genes are correlated in expression.[195] This cannot be achieved by whole cell population or tissue analysis because the signal derived from individual cell expression patterns will be averaged. RNAs expressed only in a few cells will be diluted so that their presence might not even be detected, thus making it difficult to elucidate co-regulation patterns.[196]

Single cell gene expression analysis is probably the easiest approach to obtain a comprehensive picture of the molecular phenotype of a single cell. This is due to the availability of very sophisticated and sensitive methods for the detection of RNA and the possibility to amplify the RNA amount in order to detect even low abundance RNA species.

In principal gene expression analysis can include a) the detection of certain mRNA species or the whole transcriptome in order to know which genes are transcribed in a certain cell type under certain conditions, b) the study of gene regulation to elucidate the connections of different regulatory proteins and gene sequences (such as transcription factors, microRNAs, epigenetics) in a cell and identify how they interact in order to influence gene expression, and c) the analysis of translation of mRNAs to proteins which reveals posttranscriptional regulation and numbers of proteins that are produced from one mRNA copy. This is important for functional validation of gene expression.

Already on mRNA level several methods can be used for single cell gene expression analysis that deliver quantitative and qualitative information about the presence of certain or all transcripts and that use different approaches (Table 2).

Table 2: *Performance parameters of gene expression analysis methods.*

technique	excessiblity of different transcripts	information on spatial occurence	single transcript sensitivity	speciality
RT-qPCR	about 10	no	yes	equippement from large scale RT-qPCR applicable
RNA-FISH	less than 10	yes	yes	super resolution microscopy necessary for advanced studies
hybridization microarray	several thousands (pre-amplifiaction assumed)	no	no	transcripts of high sequence similarity hardly discriminable
protein based strategies	less than 10	yes	yes	transfection required
sequencing	whole transcriptome (pre-amplifiaction assumed)	no	no	no pre-definition of relevant transcripts required

Reverse transcription quantitative real time PCR (RT-qPCR) and hybridization microarrays

Single cell RT-qPCR is a highly sensitive method that in principle allows for the detection of only one mRNA molecule. The method is quite suitable for the quantification of certain, selected mRNA species in many different single cells due to its high reproducibility and wide dynamic range.[190, 195] The cells are lysed, then, the released mRNA is transcribed into an identical DNA copy (cDNA) by reverse transcriptase, and finally, the cDNA is hybridized to a primer allowing its exponential amplification through DNA polymerase. The amplified DNA is detected in "real-time" by monitoring a fluorescence signal, either from a fluorescent intercalator or specific fluorescent tag. Since the resources and technical know-how for qPCR are widely established in many laboratories, RT-qPCR on single cells can be easily applied without high costs and efforts. For single cell analysis careful work is especially essential and any contamination in sample preparation must be avoided for an exact mRNA quantification due to the presence of only few mRNA molecules in one cell. Cell lysis should be efficient with maintaining the integrity of the RNA at the same time, and any RNA degradation in the

lysate should be avoided by the use of efficient RNAse inhibitors. Further, reverse transcription should be efficient to make sure that all mRNAs get copied to cDNA, and it must be ensured that no primer-dimers are formed or unspecific amplificates are produced during to PCR, since they can obscure the exact quantification because of the low detection limit.[190, 195] Essential information for publication of qPCR results is given in the MIQE guidelines. [197]

An important drawback of single cell RT-qPCR in comparison to whole cell population RT-qPCR is the fact that due to the non-correlated and highly variable expression of different genes in different cells normalization to reference genes cannot be applied. This makes accurate quantification for comparison between different cell types difficult. A solution could be the addition of a known concentration of spike reference mRNA[190].

Further, with single cell RT-qPCR only 5 to 10 genes can be analysed in one cell without pre-amplification due to the relatively small sample size.[190] This might be overcome in future by using digital PCR. With that method single cDNA molecules can be detected. The sample from a single cell is diluted within miniaturized microfluidic devices to yield either one strand or no cDNA in one cavity where sensitive, automatic and reliable high-throughput PCR is carried out. [198] Another recent advancement of expression analysis aims at the transcriptome-wide analysis of the gene expression profiles by incorporating an universal PCR priming sequence via tagged priming and template switching.[199]

To date, microarray analysis offers a good way to study thousands of different mRNA species in one cell at the same time. This usually occurs through hybridization of the cDNA to specific oligonucleotide probes bound on a chip, and the resulting fluorescence-signal as read-out for quantification. Compared to PCR the disadvantages are possible cross-hybridizations and the low dynamic range, which can lead to falsification of the results.[196] For both PCR and hybridization microarray, the need for pre-designed oligonucleotides and a priori knowledge about the mRNA sequences makes it difficult to detect unknown RNA species as well as alternative splice variants that are only expressed under certain conditions.[196]

RNA Fluorescence In-Situ Hybridisation (FISH)

The principle of FISH is to hybridize a fluorescently-labelled oligonucleotide probe to nucleic acids, DNA or RNA. As for PCR a nucleotide sequence unique for the gene or gene product of interest compared to the rest of the genome or transcriptome is required for a successful FISH experiment. Specificity results from probe-target complementarity, thus, the definite base pairing between adenine and thymine on the one hand and guanine and cytosine/uracil

on the other hand known from DNA amplification and its transcription into RNA is exploited. Possibilities to label probes directly or indirectly are diverse.[200] Originally FISH was developed to visualize certain DNA regions; oligonucleotides spanned several hundreds of basepairs (bp) for this purpose. In this field it rapidly evolved to paint whole chromosomes.[201] Concerning mRNA analysis the key argument striking for FISH against PCR-based strategies is the delivery of spatial information. mRNA tracking from nascence to degradation would be, in principle, possible that way and it also makes single cell resolution an intrinsic feature.

RNA and DNA FISH can be performed next to each other, and also a combination with immunofluorescence labelling is possible. In a recent contribution Chatre and co-workers studied mitochondrial diversity in mammalian cells with respect to transcription and replication in that way and reported remarkable differences not only between individual cells but even between several mitochondria in one cell [202]. This gives an intriguing proof for the relevance of down-sizing experimental setups to single cell resolution.

The detection capacity of RNA FISH had been very limited until the end of the last century, due to fluorescence background noise. Singer and colleagues were the first who labelled short oligonucleotide samples (~ 50 bp) with up to five fluorophores to increase the number of fluorophores on a single mRNA target in order to detect it above the background.[203] However, the technique suffered from reduced binding specificity resulting from the high fluorophore load relative to the number of nucleobases and a difficult separation of completely fluorophore-conjugated oligonucleotides against only partly conjugated ones.[204] In 2008 Raj and colleagues described the possibility to shorten the oligonucleotide strand further, down to ~ 20 bp. This allowed the hybridization of even more probes to one target mRNA and therefore, the coupling of only one fluorophore to the 3' end of each probe. The reliability of probe binding was remarkably improved that way and diffraction limited single transcript visualization became reality. The simultaneous analysis of the expression of three different gene transcripts as well as the applicability in whole organisms was shown by these authors.[205] Sample barcoding, as suggested by Singer and co-workers, could further increase simultaneous detection capacities.[200, 203] Nowadays, single molecule RNA FISH serves to gain insights into reprogramming of fibroblasts to stem cells[206] or to assess stem cell markers in mouse intestine [207], just to name a two examples. Nevertheless, the shortness of recent oligonucleotide strands impairs hybridization selectivity. This is because of the increased probability of binding to highly similar or even identical nucleobase sequences in mRNAs belonging to different genes. The problem of high sequence similarity is circumventable to some extend by locked nucleic acids (LNA).[208] An approach to separate targets with single

nucleotide difference was described by Larsson and colleagues[209]. The detection of single miRNA with a single single-labelled LNA probe has also been reported.[208] For more detailed information on single transcript FISH the review by Itzkovitz and van Oudenaarden is recommended.[204]

Fluorescent protein-based strategies

By using fluorescent fusion proteins, gene regulation mechanisms in living cells can indirectly be assessed over time. One example is the genetic engineering of fluorescent proteins that are fused to a repressor protein and a target protein, respectively. The regulation of the expression of the target gene by that repressor protein can then be investigated by following the fluorescent signal intensities.[210] Not only transcriptional regulation but also local translational regulation can be analyzed by using fluorescent protein engineering technology. Transfection of cells with mRNAs encoding fluorescent proteins can reveal the translational activity of these mRNAs in certain subcellular regions in polarized cell types. [196]

However, fluorescent fusion proteins tend to diffuse rapidly in the cytoplasm and can have a high stability, thus, making it difficult to study the temporal and spatial behaviour of gene expression in specific subcellular regions.[211, 212] Firefly luciferase offers a good alternative due to its short half-life making it very suitable to monitor fluctuations in gene expression. [212] Further, novel techniques were developed that allow direct fluorescent measurement of gene expression of single mRNA molecules.[211] Such a technique is the MS2 tagging system, a reporter system using transfection of a cell with two plasmids: one plasmid codes for the fluorescent-tagged capsid protein of the MS2 virus and the other plasmid contains the gene of interest with MS2 binding sites that allows binding of the MS2 protein to the RNA stem loop structure of the binding site.[211-213] Another example is the hybridization of mRNA using molecular beacons, single-stranded oligonucleotides tagged with a fluorophore and a quencher, that separate and thus give a fluorescent signal as soon as the oligonucleotide binds to the target RNA.[211, 214] Further, modified and new, improved fluorescent proteins with higher stability, less cytotoxicity, photoswitchability and near infrared excitation wavelength have been developed in the recent years allowing to monitor gene expression *in vivo*[212]. Together with the advancements in super-resolution microscopic techniques below the diffraction limit (see section III.8.4), these methods have great potential for high-resolution single-molecule studies in order to analyse the dynamics of gene regulation in single cells in the future.

RNA-sequencing

RNA sequencing (RNA-Seq) with the currently available next-generation sequencing approach is probably the method of choice to analyze the complete transcriptome of a single cell [196, 215]. To ensure that no sequences are missed the RNA must be pre-amplified either through exponential amplification by PCR or through linear antisense amplification using primers with a binding site for T7 polymerase that ensures the maintenance of the relative mRNA amount. [215] Although the computational analysis and sequencing chemistries still need to be improved, the existence of specific algorithms for RNA-Seq data analysis already allows the extraction of usable information from the data.[196, 215] This and the additional functional validation of the transcriptome data are important for future applications such as the identification of potential therapeutic drug targets for the treatment of certain diseases [215, 216] or dissecting the transcriptome heterogeneity of mouse oocytes in order to understand the underlying developmental biology for advancements in stem cell research. [217] Furthermore, molecular labelling of each single mRNA in a sample with unique molecular identifies (UMIs) allows absolute and exact quantification of all mRNA copies by RNA sequencing because the quantitative information is reliably maintained during amplification by PCR.[218] This efficient combination of qualitative and quantitative mRNA determination holds the potential to powerfully advance transcriptome analysis in single cells.

Integration of gene expression analysis approaches

Currently, a decision has to be made between high-dimensional information on gene expression (array-based strategies or RNA-sequencing) where the cell has to be destroyed in advance and spatially resolved data on mRNA presence (RNA FISH or fluorescent protein-based approaches), that detect only few mRNA species at a time. Transcriptome wide and spatial information are combinable only if several cells are included, either in a bottom-up or a top-down way. Bottom-up means to start with knowledge on a confined number of transcripts and to build up a whole network, e.g., relying on interactions in a signalling cascade. While top-down refers to the in depth study of several transcripts whose relevance is concluded from transcriptome wide information derived at the beginning. For a confrontation of both strategies see Tischler and Surani [189].

Gene expression analysis on the single cell level was shown to advance studies that correlate small genetic variations with gene expression differences by showing differences not only between but even in the same individual [219] or that directly assess small nucleotide variations

in RNA transcripts to analyze allele expression differences in single cells[220]. Ultimately, this may help to understand individual differences in organ functions and associated diseases.

V.2 Single cell proteomics

Although measurement of mRNA can already give a good hint on the protein expression diversity in a cell, it cannot tell about the quantity, location, protein-interactions and post-translational modifications of proteins [221]. This can be realized by the direct measurement of the protein composition in a cell. Whereas plenty of methods are available for efficient cell population protein analysis, single cell protein analysis is much more difficult, because of the small amount of proteins in a single cell that needs highly sensitive methods for detection. Especially the elucidation of the whole proteome is challenging due to its high complexity resulting from different organelle locations of the proteins (membrane-bound, nuclear, cytosolic proteins etc.), diverse post-translational modifications, protein translocations, and differing levels of activity [221].

Probably the most suitable method to analyse the whole proteome in this regard is mass spectrometry (MS), since it is label-free and can basically detect all proteins, post-translational modifications and peptides in one cell [221, 222]. In addition, MS-based proteomics allow the identification of endogenous protein interaction and modification during signalling [223]. For example, MALDI-MS (section III.7) and electrospray MS (see also section V.4) have been used for the analysis of certain proteins or peptides in single cells already. [224-226] Though, mass spectrometry has the disadvantage that it is not sensitive enough yet to allow detection of low abundance proteins. However, this can be improved by selective enrichment of cell subpopulation or cell fractionation, for example using microfluidics or FACS (see also section VIII.3/4). [221, 222]. Progress has been reported with respect to the successful proteome analysis of such pre-sorted subpopulations to answer specific questions (see Altelaar *et al.* [222] for a review of the studies).

Methods that employ separation of proteins are readily used on bulk protein samples; however they are difficult to apply on small protein samples from single cells. Microfluidic and capillary electrophoresis are able to overcome this problem [221], and successful attempts in this direction have been made, e.g. there are microfluidics available now to quantify low-abundance proteins. [227]

In contrast to proteomics, studying single proteins in single cells is more advanced: methods like flow cytometry or mass cytometry use specific antibodies and allow to study several proteins at one time (see also section VIII.3). Fluorescence-based arrays with antibodies

bound to the surface can bind several proteins at the same time. This has been used to quantify intracellular signalling proteins in a cancer cell line [228] or to detect cytokines secreted from single cytotoxic T cells and other cells [229, 230] or mononuclear cells [231]. Further, a combination with microfluidics to allow trapping, lysis and protein measurements in one system has been established and optimized recently as so called microfluidic antibody capture chips [232, 233].

In contrast to whole proteome studies, there are already numerous and multifaceted studies on the analysis of only a few, specific proteins in single cells. Those examples won't be discussed here, instead the reader is referred to the review of Wu and Singh.[221]

V.3 Single cell metabolomics

Single cell metabolomics addresses fundamental biological questions and is capable to observe metabolic phenomena in heterogeneous cell populations [234, 235]. The cell metabolome usually includes all intracellular and membrane-localized small molecules/metabolites with a molecular mass less than 1 kDa, e.g. lipids and carbohydrates. The metabolits can be exogenous, originating from outside the cell (as known as xenobiotics), or endogenous. Metabolites are involved in many intracellular functions and provide information of the physiological condition of the cell. Over the past few years metabolomic approaches developed rapidly and a number of useful databases, which store, manage and analyse the metabolomics data, occurred. The review by Go provides an overview of the recent progress in databases employed in metabolomics. [236] The most common techniques to measure the untargeted metabolome of tissue and other biological samples is the separation combined with mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy [237]. Beside these detection techniques, the analyte extraction from the target cell becomes a crucial experimental step [73], because there is only a minute quantity of analytes in a single cell. Even the detection limit has been lowered from femtomoles to a low attomole range for single cell metabolomics [238]. Quantification is still problematic due to the need of conserving the original metabolome, which is often difficult because of the presence of enzymes in the sample and the fast metabolic turnover rates. [239] Microfluidics, gas and liquid chromatography and capillary electrophoresis as separation techniques in combination with detection methods like (laser induced) fluorescence or MS are the most promising techniques for single cell metabolome studies.

Mass spectrometry (MS) is an indispensable research tool in metabolite and peptide characterization. The capability to detect metabolites on a single cell level was partially

described in section III.7 concerning mass spectrometry imaging (MSI) techniques. The workflow during MS measurements is almost the same: The analytes are transferred into the gas phase, ionized, separated and analysed by their mass-to-charge ratio and finally detected. In addition to MSI methods, great affords in recent metabolomics studies based on MS techniques were made (reviewed by several authors[73, 234, 237]). Furthermore, Heinemann and Zenobi give an interesting overview of the current MS-based approaches for single cell metabolomics relating to their advantages and disadvantages [239].

One example for the increasing capability of single cell metabolomics via MS was shown by Nemes and co-workers by combining intracellular small volume samples with capillary electrophoresis (CE) and electrospray ionization (ESI) MS analysis. Thereby, over 300 distinct peaks were obtained in individual neurons. Furthermore, the identification of 36 intracellular metabolites and their quantitative analysis highlights the versatility of this technique[240]. For a detailed description of the CE-ESI-MS measurements of multiple metabolites including classical neurotransmitters (e.g. acetylcholine, histamine), energy carriers (e.g. adenosine) and osmolytes (e.g. betaines) among others in individual neurons from the sea slug (*Aplysia californica*) and rat (*Rattus norvegicus*) see the protocol from Nemes *et al.*[241] Intracellular sampling and high resolution ESI-MS detection of metabolites from single plant cells was done by Lorenzo Tejedor *et al.* [242] Oikawa demonstrated the utility of a large single cell model for an investigation of the metabolome and determined functional changes in the metabolite profiles of subcellular regions via CE-MS.[243] For more details concerning CE-MS see the review by Klepárník. [244]

In addition, flow cytometry (FC) with an extremely high throughput in single cell measurements in combination with MS detection offers the possibility to increase the number of independent measurement channels[237]. Mass cytometry, FC- Inductively coupled plasma (ICP)-MS, can use molecular probe labels containing rare earth elements[245]. Recognition of proteins with specific antibodies containing these elements, e.g. ytterbium 171 or neodymium, and their analysis via ICP-MS allows cellular antigen detection. Furthermore, a simultaneous quantitative analysis of more than 34 parameters, e.g. binding of 31 antibodies[246], cell viability, DNA content, and relative cell size at up to 1000 cells/s becomes possible[237]. Mass cytometric detection of metabolites as well as drugs including cases where the marker atom is incorporated into the analyte molecule itself, instead of in the affinity probe[247].

The combination of microfluidic devices and MS is seen as the method with the highest potential to deliver relevant data for systems biology. Thereby, single cell organisms are processed on the microfluidic chip for a quenching, lysis, and separation of the metabolites

from the other cell components. Afterwards, a transfer to the MS devise takes place. Coupling of the microfluidic device to an ESI-MS is also possible. [239] The newest approach is the single cell elemental analysis via femtosecond laser ionization time-of-flight MS. [248]

Following mass spectrometry (MS), nuclear magnetic resonance (NMR) spectroscopy is the second most common technique to detect metabolites, however, so far mainly established for multicell analysis. Nevertheless, NMR can also be applied *in vivo* [72, 249]. The minimal sample preparation offers high-throughput studies. By its information-rich, reproducible and highly reliable character it is also capable to detect low-molecular-weight metabolites. For more details concerning metabolomics by NMR spectroscopy see the following reviews by Zhang *et al.* [250] and Gebregiworgis and Powers.[251]

However, due to its relatively low sensitivity NMR spectroscopy has reduced application possibilities on single cells. The studies by Grant *et al.*[252] and Lee *et al.*[253], which were already mentioned in the section III.6 concerning NMR imaging, are the most prominent investigations of NMR spectroscopy applied on single cells. Furthermore, achievements with new small-volume probe technologies, e.g. microcoils and microslot waveguide probes, enhance the detection limit and therefore potentially allow the characterization of cell-sized samples [73]. In conclusion, further efforts are needed to render NMR metabolomics applications of single cells.[234]

Furthermore, fluorometric metabolomics assays exist, which are generally based on the presence of fluorescent tags and a readout with an established technique, e.g. with fluorescence microscopy. The key advantages of fluorescence detection of intracellular metabolites include the high sensitivity, the capability to perform concentration dynamic studies, the nondestructive character and the high-throughput. Nevertheless, only a few metabolites can be analyzed directly in single cells by autofluorescence. In most of the cases a difficult labelling is required limiting the application capability. Especially fluorescent probes, which are expressed in living cells, can lead to an alteration of the native physiological status on the metabolome level of the cell - a further limitation of its applicability. Although nanosensor probes can be specific for different analytes, the number of simultaneously detectable components is limited as well. [234]

Electrochemical detection shows a high sensitivity making it capable for single cell analysis, even for quantitative studies. A label-free detection of intracellular and extracellular metabolites is possible. However, only electroactive species can be analyzed, which makes the electrochemical methods applicable only to targeted studies of metabolites in single cells.

Nevertheless, monitoring of various physiological processes, e.g. release of catecholamines and oxygen, could be measured.[234]

Autoradiography and spectroscopic methods (Fourier transform infrared and Raman spectroscopy, see section III.4) are also applicable to analysis metabolites in single cells.

In summary, single cell metabolomics is still at the beginning of development and no in routine used method, as it is for macroscopic samples. Overcoming the listed limitations is the main challenge for highly sensitive, comprehensive and quantifiable single cell metabolomics assays. In most of the cases different techniques are available and can be also combined. One example is the detection of nitric oxide (NO), which is involved in a wide range of biological functions. There is an increasing interest in following nitric oxide synthases (NOS) activity directly by monitoring NO production, its function and metabolism [254]. Therefore, fluorescence imaging, CE–laser induced fluorescence and NO selective electrodes were successfully used for single cell NO production. Other methods, e.g. chemiluminescence, gas and liquid chromatography-MS were also applied, but are not as commonly employed for cellular and subcellular NO levels. In addition, the analytical techniques complement each other, e.g. electrochemical detection was combined with fluorescence imaging to study NO production in living systems with spatial and temporal specificity [254].

VI. Cell physiology and mechanics

Biophysical properties of cells can serve as label-free markers of the cells' physiological state. Understanding the changes in biophysical properties in single cells can contribute to understand human diseases. [255]

VI.1 Electrical properties

Electrical properties of a cell depend on the morphology of the cytoplasmic membrane, its lipid bilayer composition, thickness, and size as well as the ion concentration in the cell. Early models depicted the cells as a spherical body of cytoplasm confined by a thin dielectric membrane. [255]

Electrical properties of cells can serve as the basis for counting, trapping, focussing, separating and characterizing single cells. [255] Dielectric properties of a cell can be assessed in a non-invasive and label-free manner via alternating current (AC) electrokinetics and impedance measurements.[255, 256]

AC electrokinetic methods study the behaviour of the individual cells in an inhomogeneous electrical field. The cells experience a force and move (dielectrophoresis, DEP). If the phase

of the electrical field is anisotropic the cell will also experience a torque and start rotating (electrorotation, ROT).[257] ROT is the only method which can determine intrinsic electrical properties of the cell such as specific membrane capacitance and cytoplasm conductivity and permittivity. [255] It was successfully applied to characterize leukocytes and human cancer cells.[255] Drawbacks of this technique are the slow speed (30 min per single cell) and the limitation to low conductivity sucrose buffer solution. Physiological buffers cannot be used due to their high conductivity. [255]

The principles of impedance analysis of particles and the state-of-the-art in the field of microfluidic impedance flow cytometry can be found in a review by Sun and Morgan[258]. Impedance analysis can be carried out on flowing as well as on trapped cells. The Coulter counter was the first cytometer which could count and size individual cells based on their electrical properties (different resistance than the surrounding conducting fluid). It is still the dominating approach in the field[258] and was implemented into haematology analysers which are nowadays used in the clinics.[255] Miniaturized microfluidic Coulter counter entering the market now. However, those are unable to characterize the cell's electrical properties.

Microfluidic single cell impedance flow cytometry can reveal physiological information such as viability and membrane potential changes [259] as well as membrane capacitance and cytoplasm conductivity.[260] It has already been successfully used to obtain a differential count of leukocytes. [255, 261-263] However, up to now, it is difficult to correlate the observed electrical property changes to physiological changes in the cell. [255]

Microelectrical impedance spectroscopy (μ-EIS) probes the current response across a trapped cell.[255] Different techniques have been developed to trap the cells. Dynamic monitoring of electrical properties of the cell during growth or interaction with other substances is possible using either a microhole chip design or microelectrodes where single cells are directly grown on electrode holes or the electrodes themselves, respectively. [264-266] Different designs of such electrode traps are also possible. [267] However, the obtained parameters still depend on electrode size, cell trapping mechanism, cell volume and interactions between the cells.[255]

VI.2 Ion concentration, channel proteins and patch clamp

The effective ion activity in and around a cell plays an important role in determining the membrane potential and the rate of physiological interesting reactions. Therefore, the cells have the ability to actively modify the ion distribution by membrane channels and transporters. There are different methods to determine the ion concentration and follow the ion transport across the membrane involving radio-labelled tracers, ion-sensitive fluorescent indicator dyes, and ion-selective microelectrodes. [268] Ion-selective microelectrodes are glass

capillaries with an ion-selective liquid membrane at the tip. These electrodes can be placed at the cell surface or inserted into larger cells. In combination with vibrating probe technologies, these electrodes can be even used to measure net ion fluxes. [268] (see also section VI.3) For small single cells ion-selective microelectrodes are not the method of choice, but rather fluorescent dyes are used instead.

The gold standard method to study cellular ion channels is patch clamp which can provide highly accurate and rich information on ion channel activity and action potential via direct measurements. Whole cell patch clamp capacitance measurements can be used to study single exocytotic events in neuroendocrine cells, and was also combined with voltage clamp pulse stimulation and with stimulation by photorelease of caged calcium. [269] Further, ionic conductance in red blood cells was found to be mainly involved in pathophysiological scenarios.[270] Many modifications of the original patch clamp technique led to improved efficiency and previously unavailable data which is extensively used in cardiac cellular electrophysiology. [271] The application of electrophysiological methods to study transporters in native cellular membranes was recently reviewed by Grewer *et al*. [272] In combination with perturbation deep mechanistic information can be obtained.

In recent years, several attempts were carried out to improve throughput and make the technique available for characterizing drug – ion channel interactions. Innovative 'lab-on-a-chip' microtechnologies that modify design, fabrication, as well as enable microfluidic integration have been reviewed by Yobas. [273]

VI.3 Assessment of further physiological properties

For the sensing of biological relevant molecules such as O_2, NO, H_2O_2, ascorbate, glucose, dopamine, glutamate and ethanol microelectrode based approaches have been developed. [274] Further developments in this field do not only enable the determination of static concentrations, but also of the dynamic physiological flux. As this approach has been developed by several groups in parallel, it has many different names, such as vibrating probe, **self-referencing microelectrode**, microelectrode ion flux estimation and microelectrode flux estimation techniques.[19, 275, 276] Different variants include self-referencing amperometry where the analyte is either reduced or oxidized and self-referencing biosensors where electrochemically coupled enzymes are involved. [277]

While the electrode based techniques are limited to detect concentrations in the close vicinity of the cell, intracellular probes based on luminescence quenching are available for the **detection of oxygen** concentrations as well.[179] Very popular are luminescent metal (Pt, Pd, Ir, Ru) porphyrins whose luminescence lifetime and intensity can be quenched by molecular

oxygen. [278] Also π-conjugated polymer nanoparticles have been proposed as fluorescent oxygen sensors. [279] These can be internalized into the cell by phagocytosis (especially investigated with macrophages), transport systems, such as microinjection, electroporation [280], liposomal transfer, facilitated endocytosis [281], gene guns or with the help of special ligands that allow cell penetration [172, 282-284]. Application examples as well as a discussion of advantages and shortcomings of the different techniques have been given by Dmitriev and Papkovsky. [278] Multichannel biochips are under development for a parallelization of these techniques with the aim of achieving a higher sensitivity [169].

Calcium is an important mineral and **calcium ions (Ca^{2+})** play an important role as signalling "molecules". Therefore, elaborate techniques exists that can reveal calcium concentration and distribution inside living cells using confocal and two-photon fluorescence imaging (section III.8). Small molecule fluorophores that can chelate calcium ions or genetically encoded calcium indicators based on green fluorescent protein (GFP) are typically applied. [285] Calcium imaging is especially used to study neurons and neural activity. An extensive review is given by Grienberger and Konnerth. [286] Successfully calcium imaging in whole organisms was demonstrated within the commonly studied model organisms (see also see also section VII) *Danio rerio* (zebrafish) [287] and *Drosophila melanogaster* (fruit fly). [288]

Organic metal complexes have the potential to sense several other analytes as well. However, so far most of these studies were carried out under non-physiological conditions and cellular experiments have been limited to up-take experiments. [289, 290]

Maintaining the right pH in the cell and in the organelles is essential for the proper function of the cell. The **intracellular pH** can range from 4.7 in lysosomes to around 8 in mitochondria [291, 292]. Measurements of intracellular pH mostly utilize pH sensitive organic probes or fluorescent proteins that can be functionalized for specific cellular compartments. [292-294] Another approach uses SERS nanosensors [295].

Several other assays have been developed to detect and quantify analytes inside a single cell. The technology spectrum ranges from fluorescence to radiometric and enzymatic approaches employing different labelling and direct and indirect detection strategies. A detailed review of those techniques is beyond the scope of this review.

VI.4 Cell mass and water content

Refractometry, a technique relying on the refractive index, of a cell is done in a label-free way. In first approximation, the refractive index is dependent on the partial concentration of molecules in the cell. It has to be determined by a kind of titration of immersion liquid containing a known solid concentration against the cell compartment of interest. Under a

phase contrast microscope the compartment will vanish as soon as the solid concentration in there equals that in the immersion liquid. Already in the 1950s refractive index measurement has been successfully introduced to cell biology for the study the cell mass and water content.[296] Thus the refractive index serves to assess key physiological parameters. Refractometry has received certain attention during the last decade. Hilbert phase contrast microscopy which is used to acquire the refractive index map has been coupled to confocal reflectance microscopy which serves to extract information about the physical thickness of the specimen in order to improve the accuracy of refractive indices.[297] Furthermore, a tomographic three dimensional mode was introduced by Choi and co-workers that at the same time avoids keeping the cells in non-physiologic immersion substances.[298] The possibility to assess the chromosome mass by a derivative optical tomographic approach in a quantitative manner has been described recently.[299] Related to refractometry, Reed and colleagues implemented interference microscopy into this approach and studied the effect of drugs on a cancer cell line by quantifying the cell mass in a time dependent and high throughput manner.[300] A different setup, called spatial light interference microscope, was used by Mir and colleagues to measure the cell mass in relation to the cell cycle. Their development is applicable as an ad-on for a commercial microscope system allowing for combination of mass assessment and fluorescence acquisition.[301, 302]

Intrinsic physical properties of the cell, such as refractive index, composition, size, and deformability determine how a cell will react in an optical gradient field. Time-of-flight (TOF) optophoresis is used to probe the speed differences between different cells and by this to distinguish cell lines and drug-treated cells [142].

Suspended microchannel resonators were developed to determine the mass of a bacterium in water with sub-femtogram resolution [303]. Channel height limitations have been overcome so that now also eukaryotic cells can be weighted in such channels. Over 30 minutes the growth of individual cells (*Saccharomyces* sp. and mouse lympohblasts) could be followed by measuring the buoyant mass. Observing individual cells it was found that heavier cells grow faster than lighter cells. [304]

Furthermore, different mechanical resonator systems can be used to determine the mass of a cell placed on the surface of such a resonator [305]. The density of a single living cell can be measured by recording the mass of the cell of interest in two fluids with different density. [255] This was successfully applied to distinguish red blood cell associated disease such as malaria, sickle cell disease and thalassemia. [306]

VI.5 Mechanical properties

Cellular membranes, the cytoskeleton composition (both, structural proteins and cytoskeleton-associated proteins play a role) as well as size and density of the nucleus determine the mechanical properties of the cell such as its deformability. Local measurement techniques, such as atomic force microscopy (AFM, see section III.2.1) [307], magnetic bead-based rheology or optical tweezers (see section VIII.1) [308, 309] and micropipette aspiration [310, 311] make it possible to probe mechanical properties of individual single cells also in liquids which resembles their natural environment. A summary of the functional range of different techniques to probe cellular mechanics is given by Loh *et al*. [312] A useful parameter to describe the elastic properties of cells is Young's modulus which, however, is dependent on various other factors. Therefore, same experimental conditions are crucial for comparability [313]. Probing intrinsic biophysical markers, such as elasticity does not require costly labels or extensive sample preparation.

Most research was done with cells for which deformability is of physiological relevance. Those are red blood cells (RBC), leukocytes and also cancer cells (potential circulating tumour cells) that have to squeeze through small blood vessels. Characterizing the cell's stiffness and deformability might give insights into different cell states and several human diseases, such as cancer, malaria, leukaemia, sickle cell disease, sepsis, hereditary spherocytosis, and diabetes. [255] Several studies proved alterations in mechanical properties such as cellular deformability to be useful to differentiate non-malignant and malignant cells. [313]

A simple way to squeeze cells is to force them through constriction channels which have a smaller diameter than the cells. High speed imaging can be used to follow transit time, elongation and recovery time. Electrical impedance measurements give transit time, impedance amplitude ratio and impedance phase increase. When only electrical readout is necessary the technique can be as fast as 100 cells per second. [255] Fluorescence measurements of labelled cells in the constriction channel can help to correlate mechanical deformability with already established cell surface markers. [255] One technical problem that might affect the measured values is that the friction between cell membrane and channel surface cannot be determined yet.

In order to study the deformability of soft and flexible cell types like erythrocytes, fluid shear stress in larger capillaries can be used. [255] Furthermore, optical stretchers were used to characterize the deformability of RBCs [314], human cancer cell lines and patient's oral squamous cells. [255] A dual beam trap acting as a cell stretcher was used to create controlled

cellular deformation [315] and study the viscoelastic properties of the cell membrane in red blood cells. Malaria-infected RBCs were found to have increased rigidity due to the internalized parasite *Plasmodium falciparum*. [316]

When hydrodynamic stretching is applied to the cells, they are completely surrounded by liquid and do not have contact to the channels. High strains can be exerted on the cells which are easy to visualize. Gossett *et al*. successfully demonstrated the potential of this technique for the characterization of pleural fluid to determine leukocyte activation and cancer malignancy. [317] However, costly and bulky high speed cameras are needed that produce huge image data which require high computational effort for data analysis. Other potential applications of mechanical biomarkers in medicine are summarized by Di Carlo [318]. However, so far biomechanical markers hardly made it to clinical and biological applications. Further research needs to be done to better understand cell deformability changes as a function of environmental conditions and to improve the currently only poor correlation between cell deformability and the widely established biochemical markers. [255] Microfluidic developments will help to automate the analysis and enable high throughput. The different mechanical stimuli that can be implemented and probed in microfluidic systems to assess the cell deformability, including working mechanism, key observations and throughput have already been reviewed. [255, 319] They include, e.g., electroporative flow cytometry, DEP force as well as compressive forces applied through a thin membrane. The latter can be utilized to monitor cell viability and to induce mechanical lysis and in further modifications to reveal information about the viscoelastic properties of cells. [320]

The formation of bulges on the cellular membrane can be correlated to the cytoskeleton quantity inside the cell and used to distinguish breast cancer cells and normal cells [321].

VI.6 Binding and intracellular interactions down to a molecular level

In the previous sections a wide range of techniques has been already discussed that are able to investigate cell-substrate or intercellular interactions. Atomic force microscopy (AFM, section III.2.1) can measure inter- and intramolecular interaction forces with pico-Newton resolution. It could be successfully applied to measure interactions at the single-molecule level, e.g. to follow fibrinogen-platelet binding and fibrinogen-erythrocyte binding interactions which have relevance during cardiovascular disease. [322]

To monitor *in vivo* single molecule interactions, such as protein-protein interactions at the cell surface, single-molecule fluorescence resonance energy transfer (FRET) (section III.8.2) and fluorescence correlation spectroscopy (FCS) (section III.8.3) can be used [323, 324]. Further

single-molecule mechanical assays are proposed to measure the in situ binding kinetics on the surface of live T cells. [325]

An immunohistochemical method for the detection of proteins and protein interaction is the in situ proximity ligation assay (PLA). Affinity reagents, such as antibodies, with an amplifiable DNA reporter molecule are used to visualize the protein of interest. [326-328] Steps towards automation in microfluidics have been taken recently. [329]

Surface plasmon resonance (SPR) (section III.7) combined with a special sensor and detector can be used to study the cell's response to stimuli, such as antigens, and follow the binding interactions. SPR imaging found already application for allergy screening by studying the response of human basophils to different antigens such as pollen antigens, mite antigens or sweat antigens [330] or by studying of rat basophilic leukaemia cell to immunoglobulin stimulation [171].

Another emerging field are targeted nanomaterials that are equipped with organelle-specific carriers and an effector molecule, e.g. designed nanoparticles for drug delivery. Important questions arising with such nanodeliverers are: Are there specific binding interactions? Are other parts of the cell also interacting with the target? Confocal fluorescence microscopy (section III.6.1) and high resolution TEM (section III.4) were utilized to visualize the interaction between drug-loaded nanoparticles and cancer cell nuclei. [331] Many further examples of nanoparticle-cell interaction exist.

A well-defined control can be gained over certain proteins such as light activated channels and enzymes by a technique called optogenetics. By genetic manipulation light-sensitive proteins are brought into the cell of interest which can then be switched on and off on a time-range of milliseconds. This allows the perturbation and subsequent detailed analysis of physiological processes. [332] Optogenetics is now widely adopted in neuroscience. [333]

Microfluidic developments (see section VIII.4) enable the measurement of many interaction forces of all kind, especially mechanical interactions can be characterized easily. Traction forces on microposts arrays can be used to estimate the strength of mechanical cell-substrate interactions as a function of morphology. [255] Furthermore, cellular response to external and internal forces can be followed in a time- and space resolved manner.[334, 335] Other microfluidic channels with fluid shear forces can be used to study adhesion forces of different cells, such as mammalian fibroblasts, activated and non-activated neutrophils and human breast cancer cells. [255]

A few other examples are given in other sections of this review (VI.2, VI.3, VIII) and can be found in the literature.

VII. The single cell in the multicellular organism

In a multicellular organism single cells have to act on an advanced level of cooperation. Often synchronization is of highest relevance, e. g., for productive heart muscle contraction and relaxation. Deviations may occur between *in vitro* and *in vivo* findings. An example is given by Yoo and co-workers who described differences concerning *in vitro* data and results derived from *Danio rerio* in *in vivo* studies regarding the position of the microtubule organizing complex in migrating neutrophils.[336] However, in agreement with *in vitro* data, recent publications bare intriguing variance in the molecular phenotype of cells belonging to the same tissue or sharing the same fate, respectively. Liu and colleagues described an approach based on **mRNA recognition** by fluorescence-labelled proteins to assess a cell position related gene expression heat map in *Cenorhabditis elegans* for 93 genes in 363 cells. They described differential gene expression between cells even belonging to the same syncytium in dependence of their respective lineages.[337] The single molecule mRNA-FISH approach described in section V.1 was as well shown to work in *Drosophila melanogaster* and *C. elegans*.[205] Variations in mRNA expression of a certain downstream effector (mec-3) for proper touch receptor neuron development in dependence of genetic background (alr-1 wild type and mutant) were studied in *C. elegans* larvae.[338]

Visualization and tracking of single cells in organisms becomes possible with new development in microscopic technique called **selective plane illumination microscopy (SPIM)** and its derivatives. For a review concerning SPIM see Weber and Huisken[339]. Due to special objective configurations which illuminate only a single object plane at once, SPIM avoids photodamage. In addition, time consuming scanning is evaded or at least reduced in dependence of the beam shape. SPIM application, though, requires the samples to be as transparent as possible to minimize photon scattering. It has already been used for detailed observation and visualization of neuron outgrowth during *C. elegans* development.[340] Furthermore, Krzic and co-workers tracked several cells during *D. melanogaster* development for five hours covering two cell division cycles.[341] Further improvements especially with respect to resolution, signal to noise ratio and artefact circumvention are highlighted in a recent contribution of Gao and colleagues describing their combinatorial approach of Bessel beam super-resolution structured illumination microscopy. They illustrate, e. g., *in vivo* karyotyping on the surface of a *D. melanogaster* embryo and mention the possibility to distinguish nuclei down to 20 μm in the sample.[342]

Single cell analysis in multi-cell surrounding mostly requires labelling of a cell type of interest. For a recent contribution reviewing several advanced labelling strategies see the one by Progatzky *et al*.[343].

Several **intravital microscopy** approaches were shown to be applicable for single cell observations as well. Especially **two-photon microscopy** gained relevance. It is based on the nearly simultaneous absorption of two photons by a single fluorophore. Both photons have to possess half of the energy that is necessary to raise an electron of the fluorophore to a higher energy level. Accordingly, photon wavelengths are doubled compared to single photon excitation of the same fluorophore. This has the advantage that the applied infrared wavelengths enable deep tissue penetration. The requirement of two photon absorption at once leads to intrinsic high confocality of two-photon fluorescence microscopy without the need for special optics.[344] In particular cells of the immune system have been the objective of single cell two-photon fluorescence microscopy.[345-347] As the basic review on two-photon laser scanning fluorescence microscopy the one by Denk *et al*.[344] is suggested for further reading. Concerning single cell studies by intravital microscopy a comprehensive review by Weigert *et al*.[284], containing a number of examples, is available and recommended.

A technique improving penetration depth of imaging is **photo-acoustic tomography (PAT)**. It is accordingly especially interesting for *in vivo* application. PAT exploits the transformation of an incoming electromagnetic wave to a density wave by an absorber, the opto-acoustic effect. A penetration depth of 5cm in tissue is enabled while a lateral resolution of less than 1mm is retained. For less penetration depth single cell observations are generally feasible if contrast is sufficient. [348] So far, PAT demonstrated its use for real single cell studies especially with respect to erythrocyte observations. By PAT based flowoxigraphy real-time oxygen release from erythrocytes in mouse brain was observed. [349] For a review on PAT and its subdivisions to complement optical imaging see Wang [348].

VIII Micromanipulation of single cells

Most of the micromanipulation techniques of single cells employ some sort of microfluidics because this enables an automated and user-friendly manipulation [350]. Quite often several manipulation tools are combined to comprehensively characterize and study single cells. Figure 3 schematises the most common applications which will be explained in more detail in the following paragraphs.

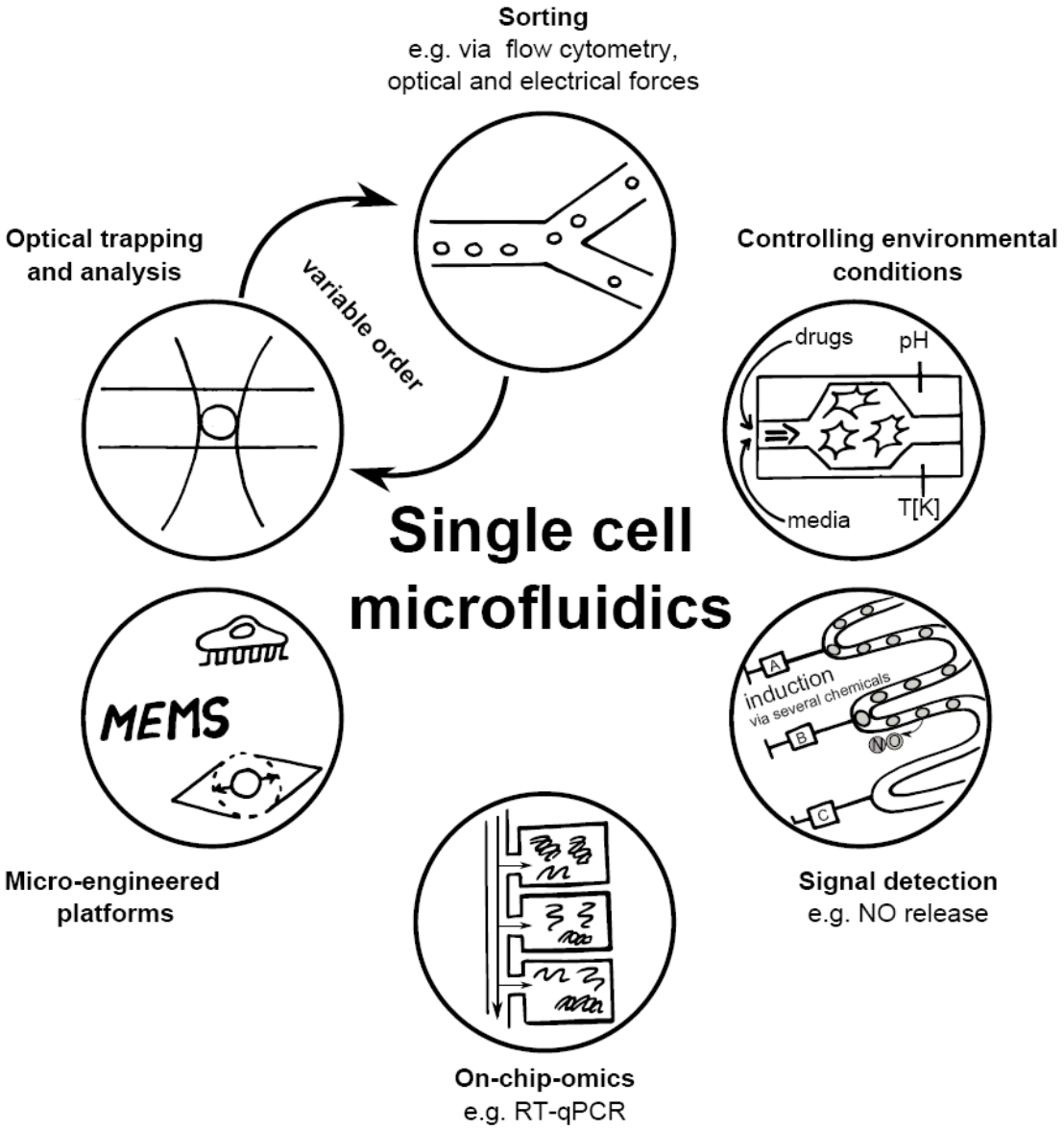


***Figure 3**: Overview of single cell microfluidics applications.*

VIII.1 Trapping of single cells

There exist several methods to trap cells in solution and to keep them stable in space. Those methods apply optical forces using lasers, acoustic or ultrasound waves, dielectrophoretic forces or magnetic fields as well as hydrodynamic flows. [351]

Optical traps and tweezers

Lasers are able to exert forces in the range of femto Newtons to nano Newtons with a force resolution of 100 aN (sub-pN) and a time resolution in the range of µs. [142] These small forces are sufficient to manipulate inter- and intracellular processes and also to move microscopic

cells. [142, 352, 353] In order to trap the cells, the surrounding medium should have a diffractive index less than that of the particle. [315] When applying lasers it is important to adjust the right parameters to avoid photodamage. Anaerobic conditions to avoid the formation of reactive oxygen species and the use of cold buffer to prevent heating effects were found to be beneficial. [142]

A single beam trap confines individual cells near the focus of the laser beam. Such traps can be easily combined with optical microscopy and various spectroscopic and optical imaging techniques such as fluorescence or Raman to further analyze the trapped cells. [315, 354] However, often traps use IR light which is not so suited for optical analysis and therefore, a second lasers is still needed.[142] Digital holographic microscopy was successfully applied to image optically trapped cells and to monitor their interaction with selectively moved particles with a temporal resolution of a few milliseconds. [355]

Dual fibre optical traps with two counter-propagating light beams cannot only trap larger cells than the single beam trap, but furthermore exert mechanical stress on the cells. Such traps can be easily implemented in microfluidic with orthogonal viewing. [315] This enables the study of mechanical properties of cells (section VI.2) or certain properties of cells under mechanic stress, such as, e.g., calcium signalling of human embryonic kidney (HEK) cells. [356]

Multiple optical tweezers can be combined to control the spatial position in 3D of hundreds of cells at a time to improve throughput. Methods to create multiple traps have been reviewed by Ramser and Hanstorp. [142]

Holographic optical tweezers can be used to create specific cell arrangements to study the influence of a certain position or interaction, [142] and to orient the cell or some of its organelles. It was, for example, possible to arrange the nuclei of different individual cells all in the same plane or move free-lying vesicles in the cytosol of NG-108 cells [357]. Movements of beads functionalized with secretory molecules towards neural cells can be even used to stimulate the neuronal growth. [358] In addition, single molecules (e.g. kinesin molecule) inside living cells can be monitored with optical tweezers. [359]

Not only lasers can be used to generate optical fields, but also arrays of gold micro-pads. When light couples to the surface plasmons, cells can be trapped with those **surface plasmon tweezers**. [360-363]

Acoustic trapping

Ultrasonic waves were also successfully used to position cells in the centre of microwells and further investigate them there. [364-366]

Other, non-optical trapping methods make use of the hydrodynamic flow (hydrodynamic trapping) which can be implemented in three-dimensional microfluidic system, or electric fields (**Dielectrophoretic trapping, DEP).** For the latter one, the electric field should not be too high in order to keep survival rates high. **Magnetic tweezers** employing magnetic nanoparticles can be used to manipulate individual molecules inside a single cell. [367]

VIII.2 Invasive manipulations

Optical scalpels: Pulsed UV-laser or NIR laser light can be focused with high precision on certain organelles or even molecules. If the energy of the laser is high enough it can act as an optical scalpel which can knock out molecules or irreversible impair organelles.[142] This was applied to damage DNA to study subsequently the DNA repair mechanisms, to photoporate cell membranes for transfection experiments (References in [142]) and to disrupt individual mitochondria in living HeLa cells [368]. Small holes in membranes induced by UV illumination can be used to study the diffusion of small fluorescent molecules and probe compartmentalization. Furthermore, targeted lysis of very small cell regions is possible with a high precision in space and time. [142]

Electroporation can be used to introduce foreign molecules (DNA, proteins) into cells by temporarily disrupting the cell membrane by a voltage shock [255]

Single cell lysis can be achieved by different stimuli: laser pulses, electrical pulses and other electrical pertubations, sonication, detergent/surfactant, and chemicals. The characteristics of those techniques regarding time, platform and denaturating influence on cellular structures and features have been reviewed by Brown and Audet.[369]

VIII.3. Separation and sorting

Optical traps can be arranged to generate optical landscapes in which cells experience different optical forces according to their shape, size and refractive index. These forces can be used for passive, light-induced separation of cells. [142]. This technique worked fine to separate red and white blood cells. For very similar cells the low sorting efficiency can be improved by binding dielectric microspheres to the cells of interest. [142]

Electrophoresis is an electrokinetic phenomenon exploiting the movement of dispersed particles in an electric field. This technique has been first employed to separate small molecules, but is now also used for single cell experiments and to profile the many different analytes in a single cell. [370] Parallel detection of more than one analyte is possible in a large dynamic detection range, opening the way to functional metabolomics studies. [371]

With capillary electrophoresis (CE) only low sample volumes are required and a fast and efficient separation of the components within single cells can be achieved.[372] The technique is portable and very versatile and can be coupled with microfluidics. Common analysis methods used in combination with electrophoresis are fluorescence (laser-induced (native) fluorescence: CE-LINF and CE-LIF, respectively), electrochemistry (EC, amperometry, voltammetry, and conductivity), and mass spectrometric detection; less common methods are based on radionuclide and nuclear magnetic resonance.[372]

Flow cytometry is a high throughput technique that uses cellular characteristics such as morphology or fluorescence (labels) to sort, count and purify cells and to determine the cellular phenotype. Detection is mostly done optically or electronically. Furthermore, flow cytometry enables quantitative analysis of protein expression, protein epitopes, protein phosphorylation state, nucleic acids, and ion concentrations in single cells. [373] However, only a snapshot in time is acquired, continuous monitoring of individual cells over time is not possible. Nevertheless, the high throughput of the technique enables to investigate the heterogeneity among a cell population. [374] Flow cytometry is already routinely used for diagnostics in haematology and immunology, as well as in cell-based basic research. Cell function and cell properties, such as abundance of special proteins [375], occurrence of reactive oxygen species, viability state and others can be determined.

Fluorescence-activated cell sorting (FACS)

The acronym FACS is a trademark and was introduced by the company Becton Dickinson, but is nowadays often used in a generic sense. [376] FACS uses the light scattering and fluorescence properties of the cells to sort them into subpopulations relying on user defined criteria[374]. Polychromatic flow cytometry uses more than 6 colours at the same time. Nowadays, with the help of fluorescence labels 18 different proteins per cell can be quantified at a rate of >10 000 cells/s. [377] This can yield deep insights into immune cell subpopulations and immune cell function.

Magnetic affinity cell sorting (MACS)

As FACS also the acronym MACS is a trademark (by the company Miltenyi). Instead of fluorescence labels magnetic beads are selectively attached to a specific antigen or cell surface marker of the selected cell subpopulation. The cell separation and purification can be carried out in positive selection mode (selected cells carry the bead) or negative selection mode (selected cells do not carry the bead).

Single cell mass cytometry

Mass cytometry is a relatively new version of flow cytometry that combines it with mass spectrometry. Theoretically, it is possible to differentiate 70 – 100 parameters in a quantitative and specific manner over a high dynamic range with high throughput (1000 cells per second).[245, 377] Instead of fluorescence labels, purified, stable (non-radioactive) isotopes of non-biological, rare earth metals (typically lanthanides) are tagged to antibodies and/or DNA intercalators and used as reporters. For analysis, the cellular material of a single cell is nebulized and analyzed with a time-of-flight mass spectrometer. Unlike conventional flow cytometry methods, single cell mass cytometry is not suitable for work with living cells and it is impossible to recover live cells back after the mass cytometry experiment. Further comparison of fluorescence based and mass cytometry can be found in the review by Bendall *et al*. [377] Applications lie in the field of immunology, stem cell research and haematology.[246]

VIII.4 From microfluidics to lab-on-a-chip

Microfluidic systems usually consist of a structure of channels (typically in the micrometer range: 10-100 μm) that can be designed individually for each experiment and equipped with functional structures (capturing elements, antibodies, electrodes, etc.). Functional assays or the combination with other experimental cell analysis techniques are thus permitted. [142] Common names for these integrated microfluidic concepts are lab-on-a-chip systems or micro-total analysis systems (μ-TAS).

Microfluidic systems have many advantages for single cells studies and therefore, have undergone a fast technical development during the last years. Only small sample volumes are necessary saving expensive reagents; increased (multi-step) integration and automation capabilities make the assays user friendly; fast response and increased sensitivity bring reliability and statistical information and environmental parameters (pH, salt concentrations, drugs, temperature and others) can be controlled precisely. [255, 373, 378] Ultimately, microfluidic systems might mimic certain *in vivo* situations in an *in vitro* setting. It is already possible to follow dynamic events and cell-cell interaction.

Many of the single cell analysis techniques described in this review have been transferred into microfluidics where sub-cellular information can be analysed by various high-contents analysis methods under defined cellular environments and stimuli. [379, 380] Single cell gene expression measurements including measurements of expression dynamics, high-throughput single cell RT-qPCR, transcript multiplexing, single cell whole genome analysis, protein analysis, signalling response and growth dynamics analysis as well as biophysical measurements could be already successfully incorporated into microfluidics. [255, 373, 381] Intracellular protein expression and the release of cytokines and effector molecules can be visualized by combining microfluidics with single molecule imaging, fluorescence imaging as well as with miniature antibody arrays. [228, 373, 382] This was shown first for bacteria but could be transferred to eukaryotic cells as well. Protein abundances were found to vary from 0.1 to 10^4 molecules per cells. Furthermore, mRNA and protein abundances seemed to be rather uncorrelated indicating a rapid degradation of mRNA. The real-time monitoring of the release of signal molecules such as NO, insulin, Ca^{2+}, neurotransmitters or histamine from single living cells in a microfluidic set-up has been reviewed. [383] Observations of single cells in microfluidic systems can further help to set up detailed kinetic models for cellular reactions and metabolics as was shown for the dynamics of glycolytic oscillations in single yeast cells. [384]

In a special version of lab-on-a-chip technologies, called droplet microfluidic, single cells are encapsulated in individual liquid containers which can act as carrier as well as microreactors. [385] Within those picoliter-sized droplets that can be generated within a few milliseconds with high monodispersity, otherwise undetectable signals of single cells, such as rare secretions, become concentrated to measurable levels. [385, 386]

Despite all the progress, in most examples, sample preparation for microfluidic analysis is still carried out off-chip on the benchtop, requiring instruments such as centrifuges to separate cells from surrounding body liquids etc. [387] This extra working step requiring extra man power and causing irreproducibility in the results so far prevented the entry of microfluidics into routine diagnostics. First attempts to integrate all sample handling steps could be shown for RT-qPCR on microfluidic chips achieving a throughput of 300 cells/run [388] and for on-chip cellomics.[389]

Applications of lab-on-a-chip systems range from basic research in proteomics and immunocytometry to haematology, human haplotyping, drug discovery and development, biosensor applications as well as stem cell and cancer research. [373, 390] Clinical impact could be generated from predictive gene expression and intracellular signalling protein signatures.

Furthermore, cellular heterogeneity could help to predict disease progression, optimal treatment strategy as well as patient survival and outcome for cancer patients. [373, 382] Furthermore, the organization of cells in complex arrays for the creation of synthetic tissue seems possible. [142]

Microelectromechanical systems (MEMS) have been developed to study the mechanobiology of living cells in microengineered platforms under close to *in vivo* conditions. [311, 391] This shall help to elucidate underlying sensing mechanisms and force transduction of cells under various mechanical stimuli which can occur in a human body, such as muscle action, heartbeat, lung action or shear stress in blood vessels, but also during cancer cell dissemination. [311] Ultimately, this will lead to a more complete understanding of how cells function. Several strategies for cell-biomaterial interactions have been developed to assure biocompatibility of the MEMS material. [392, 393] Advancements in MEMS technology allow the fabrication of cell size matching devices such as microscale electrodes and arrays for precise manipulation with spatially and temporally variable stimuli and quantitative evaluation of cellular response. [311, 391, 394]

IX. Classification or what characterizes a cell type

The amount of different data collectable from an individual cell is incredible. If the techniques available for *in vitro* studies are combined in a reasonable sequence, values on its mass, chemical composition and expression level of hundreds of genes are detectable. Making concessions at the number of gene products accessible time resolved studies on the impact of manipulations are possible. Thus the detailed molecular phenotype for each cell of interest is available in principle. But what is the excess value? What one can conclude from high end statistical analysis? As discussed before, there is no cell like the other, there is always biological variation. This biological variation is observed for all measured parameters, like size, mass, gene expression patters with different amount of transcripts and synthesized proteins, as well as different levels of metabolites or the slightly different reaction on certain stimuli. This raises the questions, how big has the difference between two cells to be in order to define a new cell type? How big is the acceptable variation in morphology and phenotype within one cell type? What is just stochasticity, or noise, in gene expression due to different transcription rates, regulatory dynamics and genetic factors?

For very different cells the belonging to different cell types is widely accepted, e.g., for healthy cells, that are integrated in the optimized function of the organism and tumour cells,

that ill-behave and perturb normal function. Several analytical methods are currently researched and established to differentiate and sort such healthy and malignant cells based on relative drastic changes in metabolism and gene expression upon the acquirement of malignancy. Polymerase chain reaction (PCR, section V.1) [395], hybridization microarrays (section V.1) [396], hyperspectral imaging (section III.8) [159] Raman spectroscopy (section III.4) [354] as well as refractometry (section VI.4) [397] were effectively applied for discrimination, to name just a few examples.

The situation of differentiating cells into different cell types gets more complex if we have a closer look at, e.g., lymphocytes and their various subpopulations. Immunologists use different surface markers to distinguish a huge variety of different cell types based on different surface marker expression patterns. Variations and a high heterogeneity are also observed if the interaction of e.g. isolated and purified natural killer (NK) cells and target cells is investigated. While some NK cells exhibit high cytotoxic activity and killing efficiency others show less or even no killing activity. Based on the interaction behaviour the NK cells could be divided into further subgroups. [398] Up to now it is not clear what determines those activity differences on a phenotypic level.

In order to elucidate the critical difference between two different cells and identify what is only a less important detail and could be assigned to biological variations, statistical data analysis methods can be applied to search for inherent structure in the data set. Unsupervised statistical algorithms, such as principal component analysis have been successfully applied to cluster results of single cell analysis methods, such as e.g. vibrational spectra and gene expression profiles. Looking at primary mouse astrocytes unsupervised analysis of gene expression profiles revealed two distinct astrocyte subpopulations. [399] In the same study, gene correlation algorithms were used to identify differences in the activity of important transcriptional pathways.

During lineage pursuance classification is also of interest, and was done in whole organism context in *C. elegans* based on gene expression analysis by Liu and colleagues [337] Again, the same intriguing question of single cell classification arises: How huge does the difference in molecular phenotype between two individual cells have to be, to assign them to be of unequal cell type? From the opposite perspective: Up to which degree of difference in molecular phenotype it is just a sign of variance of one cell type? Probably the answers to these questions won't even be the same for each cell type. There may be cells of high specialization that are highly similar one to the other, inner ear hair cells might serve as an example. On the other hand, e.g., hepatic stellate cells are known for a dramatic phenotype transition when

they switch from quiescence to activation which is reflected in gene expression[400] and vibrational spectroscopy data[41].

So far, a convincing concept how to handle the cell type term in the classification context does not exist. It is expected, that with a better understanding of single cells, their organization and function, we may better define new markers and characteristics that allow classification of cell types and a sub-types.

X. Single cell analysis for diagnostic use

Single cell research shall not only bring new insights into the mechanisms of life, but there are also some hopes that the results could be utilized in medicine. The advantage of basing the diagnostics on single cell analysis is that only minimal sample (e.g., very tiny biopsy) is required from the patient and point-of-care devices for personalized medicine might become feasible. While some of the presented single cell analysis techniques are already quite advanced and are in or close to medical applications, such as single cell gene expression analysis (see section V.1), other techniques still struggle for medical relevance, such as the biomechanical markers (see section VI.2). Microfluidic developments, advances in imaging industry and hardware based image processing are expected to help with automated analysis and higher throughput providing the necessary statistical accuracy for an ultimate clinical diagnostic application. High expectations are also raised to integrated analysis techniques where two or more of the presented techniques are combined to provide an even deeper insight into the single cell characteristics.

Key fields of application that can be seen from today are oncology, especially in the field of tumour diagnostics and circulating tumour cells, haematology, regenerative medicine (pre-implantation diagnostics and embryonic stem cell research), drug development and immunology. A few examples from those fields will be highlighted in the following.

Circulating tumour cells (CTC) can be found in the peripheral blood of cancer patients with metastasising primary tumour. It was shown that the number of CTC can give valuable insights into disease severity; change in number can report the success of therapy, while the identity of the CTC could lead to the primary tumour. Different detection methods for CTC from blood have been developed within the last years. [401] Single cell polymerase chain reaction (PCR) (section V.1) can distinguish and determine different tumour cells such as CTC, but also cancer stem cells and thus, help to find the best therapy for the patient. One of the problems with CTC is that they are only present in low number in a heterogeneous sample (the patient's blood) together with many other non-tumour cells. Therefore, high throughput

techniques are needed. Statistically meaningful data require the analysis of large cell numbers. Lab-on-a-chip technology made huge advances in the last years and now allows efficient capture, separation, enrichment, detection and count of different CTC with high throughput rates and different subsequent analysis methods. [402-405]

Not only for freely circulating tumour cells which already exist in the single cell state, but also for solid tumours and **tumour research,** single cell analysis proofed to be beneficial and to hold clinical potential. For tumour diagnosis only a very small biopsy, which has only minimal impact on the patient, has to be taken if single cell analysis methods are employed [195]. Insights into tumour heterogeneity resulting from genetically and phenotypically different tumour cells can be gained by means of single cell gene expression analysis.[195] Promising examples are the use of single cell analysis for the discrimination of colon cancer tissue from healthy epithelia by RT-qPCR [406], and the RNA-Seq transcriptome analysis of circulating tumour cells from solid melanoma. The latter study analyzed CTC heterogeneity and aimed to identify potential biomarkers [407]. Different approaches for tumour analysis on the single cell level have been reviewed by Bendall and Nolan [408]

In **regenerative medicine,** the analysis of pluripotent stem cells that are used for differentiation into certain cell types for treatment of diseases is of utmost importance. It is necessary to check their homogenous differentiation into the desired phenotype.[195] For example, concerning the heterogeneity among different neuronal progenitor cell types the transcriptome can give significant information about their cell fate and development. This is fundamental for the development of future stem cell based therapies. [409-411] Similarly, assessing the transcriptomal heterogeneity in single cells can be used to track and understand the regulation of the differentiation process of hematopoetic stem cells.[412,413] Single cell PCR is already widely used for pre-implantation genetic diagnosis to identify embryos with genetic diseases or abnormal chromosome numbers.[195] Not only transcriptome based analyses find application, but also the real-time tracking and fluorescent ratio imaging of sperm motility and energetics are carried out in automated systems in human fertility clinics and animal breeding farms [414] Manipulation of single cells with optical tweezers is conceivable for *in vitro* fertilization, stem cell research, and single cell transfection. [142, 353]

Single cell analysis can also have an impact on **drug discovery.** Integrative 'systems pharmacology' strategies use the complex response (e.g. gene expression, metabolic states or cellular phenotype) of a single cell to understand the action of a drug. Bioinformatics methods are able to extract information about the drug's targets, mechanism of action, metabolism and toxicity from these multi-parametric readouts in data-driven computational approaches. [415]

Single cell transcriptome analysis provides detailed new insights into intercellular variability of the RNA profiles and thus, helps to identify previously unrecognized drug targets. [215] For example, RNA-Seq transcriptome analysis of neuronal cells, which are highly specialized and polarized cells [196], is of great benefit for the field of neuropharmacology, because it can be used to identify new receptors and channels as potential drug targets in neurological diseases. [216]

Also mechanical characteristics of a cell (see section VI.2), such as the simple deformability which can be probed label-free, i.e., without costly antibodies, have a high potential for drug discovery and personalized medicine. Measure of cytoskeletal integrity could enable screenings for drugs interacting with actin or tubulin of the cytoskeleton, as well as the detection of resistances against those drugs in biopsied samples.[317]

Applications of single cell analysis methods in the field of **immunology** comprise, amongst others, gene profiling of induced T or B cell subsets of the adaptive immune response for the discrimination of special vaccination-induced $CD8^+$ T cell subpopulations.[416] Furthermore, several flow cytometry based assays have been developed to determine the immune status of transplantation patients. [417] Research that could translate into a better understanding of the immune response and therefore, into the improvement of therapy designs, includes the study of the immune activity of single natural killer (NK) cells against target cells (virus-infected or cancer cells). Significant differences in the number of contacts between NK cells and target cells as well as in the killing efficiency could be revealed. However, those results are still waiting for an ultimate answer. [398, 418]

XI. Conclusion

Studying single cells offers valuable insights into spatio-temporal dynamics of biological processes and interactions, even down to the molecular level. An amazing variety of different techniques and methods already exists to visualize morphology, phenotypic characteristics and gain insights into the genome of a single cell. Despite all the advances, many interesting questions remain unanswered, such as detailed signal transduction or the exact role and timely arrangement of all the involved molecules, clusters and organelles in different interaction mechanisms. In order to answer those questions further technological development and advanced data evaluation strategies are required. The combination of different single cell analysis methods is evolving which can provide global, integrated and multi-parameter information from an individual single cell which is necessary to understand the complex organization of life. Microfluidic systems already bring together different sampling

techniques to collect biophysical and biochemical information. Further promising combinations could involve super-resolution microscopy, and novel biochemical and single-molecule kinetic measurements with an improved temporal resolution. In order to efficiently utilize all the different techniques a vivid interdisciplinary exchange among specialized scientists is required to enable the physicists and engineers to design valuable tools and devices which are able to answer the exciting biological questions of life scientists. Extensive computational effort is also required to extract the important correlation and information from the data flood and the noisy and complex signals that show such high cell-to-cell variations. System biologists will contribute valuable models that describe the complex interaction networks observed in the experiments and help to understand which parameters determine the reaction and in the end the fate of the cell.

All future technological improvements have to bear in mind that the amount of sample from a single cell is limited, therefore, asking for sophisticated detection performance. In addition, since intrinsic variability between cells is large, high-throughput techniques are a pre-requisite for reliable conclusions to be made from the analysis of large numbers of individual single cells. Finally, the realization of single cell studies in the natural cell niche, such as within tissues, is desirable.

Ultimately, the results from single cell research and analysis have great future potential for application in personalized medicine with new point-of-care devices that make use of novel prognostic and predictive biomarkers. This might help, for example, to identify key mutations in cancer genes or physiological parameters of certain disease states.

Acknowledgements

Financial support by the BMBF (FKZ 01EO1002, Integrated Research and Treatment Center "Center for Sepsis Control and Care") and the DFG via the research group FOR 1738 "Heme and heme degradation products" is highly acknowledged. Furthermore, we thank Rainer Heintzmann and Marie Walde for discussion on high resolution fluorescence microscopy and figure 2.

References

1. C. Petibois, *Anal. Bioanal. Chem.*, 2010, **397**, 2051-2065.
2. R. Masyuko, E. J. Lanni, J. V. Sweedler and P. W. Bohn, *The Analyst*, 2013, **138**, 1924-1939.
3. A. S. Stender, K. Marchuk, C. Liu, S. Sander, M. W. Meyer, E. A. Smith, B. Neupane, G. F. Wang, J. J. Li, J. X. Cheng, B. Huang and N. Fang, *Chem. Rev.*, 2013, **113**, 2469-2527.
4. M. Mir, B. Bhaduri, R. Wang, R. Y. Zhu and G. Popescu, in *Progress in Optics, Vol 57*, Elsevier Science Bv, Amsterdam, Editon edn., 2012, vol. 57, pp. 133-217.
5. J. L. S. Milne, M. J. Borgnia, A. Bartesaghi, E. E. H. Tran, L. A. Earl, D. M. Schauder, J. Lengyel, J. Pierson, A. Patwardhan and S. Subramaniam, *Febs J.*, 2013, **280**, 28-45.
6. A. A. Sousa and R. D. Leapman, *Ultramicroscopy*, 2012, **123**, 38-49.
7. L. F. Kourkoutis, J. M. Plitzko and W. Baumeister, in *Annual Review of Materials Research, Vol 42*, ed. D. R. Clarke, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 42, pp. 33-58.
8. T. Yahav, T. Maimon, E. Grossman, I. Dahan and O. Medalia, *Current opinion in structural biology*, 2011, **21**, 670-677.
9. J. Pierson, M. Vos, J. R. McIntosh and P. J. Peters, *Journal of electron microscopy*, 2011, **60 Suppl 1**, S93-100.
10. I. Hurbain and M. Sachse, *Biology of the cell / under the auspices of the European Cell Biology Organization*, 2011, **103**, 405-420.
11. E. V. Orlova and H. R. Saibil, *Chem Rev*, 2011, **111**, 7710-7748.
12. A. Hoenger and C. Bouchet-Marquis, *Adv Protein Chem Struct Biol*, 2011, **82**, 67-90.
13. A. Hoenger and J. R. McIntosh, *Curr Opin Cell Biol*, 2009, **21**, 89-96.
14. C. Batchelor-McAuley, E. J. F. Dickinson, N. V. Rees, K. E. Toghill and R. G. Compton, *Anal. Chem.*, 2012, **84**, 669-684.
15. Y. F. Dufrene and A. E. Pelling, *Nanoscale*, 2013, **5**, 4094-4104.
16. X. L. Shi, X. J. Zhang, T. Xia and X. H. Fang, *Nanomedicine*, 2012, **7**, 1625-1637.
17. T. Ando, *Microscopy*, 2013, **62**, 81-93.
18. P. Hinterdorfer, M. F. Garcia-Parajo and Y. F. Dufrene, *Accounts Chem. Res.*, 2012, **45**, 327-336.
19. J. Shi, E. S. McLamore and D. M. Porterfield, *Biosens. Bioelectron.*, 2013, **40**, 127-134.
20. P. Happel, D. Thatenhorst and I. D. Dietzel, *Sensors*, 2012, **12**, 14983-15008.
21. J. Rheinlaender and T. E. Schaffer, *J. Appl. Phys.*, 2009, **105**.
22. J. Rheinlaender, N. A. Geisse, R. Proksch and T. E. Schaffer, *Langmuir*, 2011, **27**, 697-704.
23. D. Klenerman, A. Shevchuk, P. Novak, Y. E. Korchev and S. J. Davis, *Philos. Trans. R. Soc. B-Biol. Sci.*, 2013, **368**, 11.
24. V. O. Nikolaev, A. Moshkov, A. R. Lyon, M. Miragoli, P. Novak, H. Paur, M. J. Lohse, Y. E. Korchev, S. E. Harding and J. Gorelik, *Science*, 2010, **327**, 1653-1657.
25. Y. E. Korchev, Y. A. Negulyaev, C. R. W. Edwards, I. Vodyanoy and M. J. Lab, *Nat. Cell Biol.*, 2000, **2**, 616-619.
26. M. J. Lab, A. Bhargava, P. T. Wright and J. Gorelik, *Am. J. Physiol.-Heart Circul. Physiol.*, 2013, **304**, H1-H11.
27. M. Miragoli, A. Moshkov, P. Novak, A. Shevchuk, V. O. Nikolaev, I. El-Hamamsy, C. M. F. Potter, P. Wright, S. Kadir, A. R. Lyon, J. A. Mitchell, A. H. Chester, D.

Klenerman, M. J. Lab, Y. E. Korchev, S. E. Harding and J. Gorelik, *J. R. Soc. Interface*, 2011, **8**, 913-925.
28. C. C. Chen, Y. Zhou and L. A. Baker, in *Annual Review of Analytical Chemistry, Vol 5*, eds. R. G. Cooks and E. S. Yeung, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 5, pp. 207-228.
29. K. Narayan, T. J. Prosa, J. Fu, T. F. Kelly and S. Subramaniam, *J. Struct. Biol.*, 2012, **178**, 98-107.
30. S. Bergner, P. Vatsyayan and F. M. Matysik, *Anal. Chim. Acta*, 2013, **775**, 1-13.
31. I. Beaulieu, S. Kuss, J. Mauzeroll and M. Geissler, *Anal. Chem.*, 2011, **83**, 1485-1492.
32. M. M. N. Zhang, Y. T. Long and Z. F. Ding, *Chemistry Central Journal*, 2012, **6**.
33. S. Amemiya, A. J. Bard, F. R. F. Fan, M. V. Mirkin and P. R. Unwin, in *Annual Review of Analytical Chemistry*, Annual Reviews, Palo Alto, Editon edn., 2008, vol. 1, pp. 95-131.
34. X. C. Zhao, P. M. Diakowski and Z. F. Ding, *Anal. Chem.*, 2010, **82**, 8371-8373.
35. X. C. Zhao, S. Lam, J. Jass and Z. F. Ding, *Electrochem. Commun.*, 2010, **12**, 773-776.
36. G. J. Puppels, F. F. de Mul, C. Otto, J. Greve, M. Robert-Nicoud, D. J. Arndt-Jovin and T. M. Jovin, *Nature*, 1990, **347**, 301-303.
37. M. M. Mariani, P. J. R. Day and V. Deckert, *Integr. Biol.*, 2010, **2**, 94-101.
38. C. Krafft, T. Knetschke, R. H. Funk and R. Salzer, *Anal Chem*, 2006, **78**, 4424-4429.
39. M. Okada, N. I. Smith, A. F. Palonpon, H. Endo, S. Kawata, M. Sodeoka and K. Fujita, *Proc Natl Acad Sci U S A*, 2012, **109**, 28-32.
40. I. W. Schie, J. Wu, T. Weeks, M. A. Zern, J. C. Rutledge and T. Huser, *J Biophotonics*, 2011, **4**, 425-434.
41. N. Testerink, M. Ajat, M. Houweling, J. F. Brouwers, V. V. Pully, H. J. van Manen, C. Otto, J. B. Helms and A. B. Vaandrager, *PloS one*, 2012, **7**, e34945.
42. E. Brauchle and K. Schenke-Layland, *Biotechnol. J.*, 2013, **8**, 288-297.
43. C. Krafft, B. Dietzek and J. Popp, *The Analyst*, 2009, **134**, 1046-1057.
44. A. Ramoji, U. Neugebauer, T. Bocklitz, M. Foerster, M. Kiehntopf, M. Bauer and J. Popp, *Anal Chem*, 2012, **84**, 5335-5342.
45. U. Neugebauer, J. H. Clement, T. Bocklitz, C. Krafft and J. Popp, *J. Biophotonics*, 2010, **3**, 579-587.
46. C. Beleites, U. Neugebauer, T. Bocklitz, C. Krafft and J. Popp, *Anal Chim Acta*, 2013, **760**, 25-33.
47. I. W. Schie and T. Huser, *Appl. Spectrosc.*, 2013, **67**, 813-828.
48. K. Brautigam, T. Bocklitz, M. Schmitt, P. Rosch and J. Popp, *Chemphyschem*, 2013, **14**, 550-553.
49. N. Bergner, C. Krafft, K. D. Geiger, M. Kirsch, G. Schackert and J. Popp, *Anal Bioanal Chem*, 2012, **403**, 719-725.
50. M. Q. Li, J. Xu, M. Romero-Gonzalez, S. A. Banwart and W. E. Huang, *Curr. Opin. Biotechnol.*, 2012, **23**, 56-63.
51. S. Dochow, C. Krafft, U. Neugebauer, T. Bocklitz, T. Henkel, G. Mayer, J. Albert and J. Popp, *Lab Chip*, 2011, **11**, 1484-1490.
52. B. R. Wood, P. Caspers, G. J. Puppels, S. Pandiancherri and D. McNaughton, *Anal. Bioanal. Chem.*, 2007, **387**, 1691-1703.
53. C. Matthaus, S. Schubert, M. Schmitt, C. Krafft, B. Dietzek, U. S. Schubert and J. Popp, *Chemphyschem*, 2013, **14**, 155-161.
54. U. Neugebauer, S. H. Heinemann, M. Schmitt and J. Popp, *Anal. Chem.*, 2011, **83**, 344-350.
55. H. Yuan, J. K. Register, Hsin-NengWang, A. M. Fales, Y. Liu and T. Vo-Dinh, *Anal Bioanal Chem*, 2013, **405**, 6165-6180.

56. E. A. Vitol, Z. Orynbayeva, G. Friedman and Y. Gogotsi, *J. Raman Spectrosc.*, 2012, **43**, 817-827.
57. L. Rodriguez-Lorenzo, L. Fabris and R. A. Alvarez-Puebla, *Anal Chim Acta*, 2012, **745**, 10-23.
58. T. Schmid, L. Opilik, C. Blum and R. Zenobi, *Angew. Chem.-Int. Edit.*, 2013, **52**, 5940-5954.
59. L. Quaroni and T. Zlateva, *The Analyst*, 2011, **136**, 3219-3232.
60. P. Lasch, A. Pacifico and M. Diem, *Biopolymers*, 2002, **67**, 335-338.
61. J. M. Schubert, A. I. Mazur, B. Bird, M. Miljkovic and M. Diem, *J Biophotonics*, 2010, **3**, 588-596.
62. S. Boydston-White, M. Romeo, T. Chernenko, A. Regina, M. Miljkovic and M. Diem, *Biochim Biophys Acta*, 2006, **1758**, 908-914.
63. D. R. Whelan, K. R. Bambery, P. Heraud, M. J. Tobin, M. Diem, D. McNaughton and B. R. Wood, *Nucleic Acids Res*, 2011, **39**, 5439-5448.
64. S. G. Kazarian and K. L. A. Chan, *The Analyst*, 2013, **138**, 1940-1951.
65. J. P. Pezacki, J. A. Blake, D. C. Danielson, D. C. Kennedy, R. K. Lyn and R. Singaravelu, *Nat. Chem. Biol.*, 2011, **7**, 137-145.
66. C. Krafft, B. Dietzek, M. Schmitt and J. Popp, *J Biomed Opt*, 2012, **17**, 040801.
67. I. Pope, W. Langbein, P. Borri and P. Watson, *Methods in enzymology*, 2012, **504**, 273-291.
68. A. Folick, W. Min and M. C. Wang, *Curr. Opin. Genet. Dev.*, 2011, **21**, 585-590.
69. L. Wei, Y. Yu, Y. Shen, M. C. Wang and W. Min, *Proc Natl Acad Sci U S A*, 2013, **110**, 11226-11231.
70. L. Sacconi, I. M. Tolic-Norrelykke, M. D'Amico, F. Vanzi, M. Olivotto, R. Antolini and E. S. Pavone, *Cell Biochem. Biophys.*, 2006, **45**, 289-302.
71. A. H. Reshak, V. Sarafis and R. Heintzmann, *Micron*, 2009, **40**, 378-385.
72. P. R. Sander, P. Hau, S. Koch, K. Schutze, U. Bogdahn, H. R. Kalbitzer and L. Aigner, *Trends Biotechnol.*, 2013, **31**, 204-213.
73. S. S. Rubakhin, E. V. Romanova, P. Nemes and J. V. Sweedler, *Nat. Methods*, 2011, **8**, S20-S29.
74. C. E. Neumaier, G. Baio, S. Ferrini, G. Corte and A. Daga, *Tumori*, 2008, **94**, 226-233.
75. J. B. Aguayo, S. J. Blackband, J. Schoeniger, M. A. Mattingly and M. Hintermann, *Nature*, 1986, **322**, 190-191.
76. S. C. Grant, N. R. Aiken, H. D. Plant, S. Gibbs, T. H. Mareci, A. G. Webb and S. J. Blackband, *Magnetic Resonance in Medicine*, 2000, **44**, 19-22.
77. S. C. Lee, J. H. Cho, D. Mietchen, Y. S. Kim, K. S. Hong, C. Lee, D. M. Kang, K. D. Park, B. S. Choi and C. Cheong, *Biophysical journal*, 2006, **90**, 1797-1803.
78. E. J. Lanni, S. S. Rubakhin and J. V. Sweedler, *Journal of Proteomics*, 2012, **75**, 5036-5051.
79. E. R. Amstalden van Hove, D. F. Smith and R. M. Heeren, *Journal of chromatography. A*, 2010, **1217**, 3946-3954.
80. S. Chandra, W. Tjarks, D. R. Lorey and R. F. Barth, *Journal of Microscopy-Oxford*, 2008, **229**, 92-103.
81. L. E. Wedlock, M. R. Kilburn, J. B. Cliff, L. Filgueira, M. Saunders and S. J. Berners-Price, *Metallomics*, 2011, **3**, 917-925.
82. C. Quintana, T. D. Wu, B. Delatour, M. Dhenain, J. L. Guerquin-Kern and A. Croisy, *Microscopy Research and Technique*, 2007, **70**, 281-295.
83. C. Quintana, S. Bellefqih, J. Y. Laval, J. L. Guerquin-Kern, T. D. Wu, J. Avila, I. Ferrer, R. Arranz and C. Patino, *J. Struct. Biol.*, 2006, **153**, 42-54.

84. S. Chandra, in *The Encyclopedia of Mass Spectrometry*, eds. M. L. Gross and R. M. Caprioli, Elsevier, Editon edn., 2010, pp. 469-480.
85. S. Chandra, G. W. Kabalka, D. R. Lorey, D. R. Smith and J. A. Coderre, *Clinical Cancer Research*, 2002, **8**, 2675-2683.
86. G. W. Kabalka, A. L. Shaikh, R. F. Barth, T. Y. Huo, W. L. Yang, P. M. Gordnier and S. Chandra, *Applied Radiation and Isotopes*, 2011, **69**, 1778-1781.
87. J. L. Guerquin-Kern, F. Hillion, J. C. Madelmont, P. Labarre, J. Papon and A. Croisy, *Biomed Eng Online*, 2004, **3**, 10.
88. M. K. Passarelli and N. Winograd, *Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids*, 2011, **1811**, 976-990.
89. M. L. Steinhauser, A. P. Bailey, S. E. Senyo, C. Guillermier, T. S. Perlstein, A. P. Gould, R. T. Lee and C. P. Lechene, *Nature*, 2012, **481**, 516-U131.
90. R. Ait-Belkacem, L. Sellami, C. Villard, E. DePauw, D. Calligaris and D. Lafitte, *Trends Biotechnol.*, 2012, **30**, 466-474.
91. J. A. Hankin, R. M. Barkley and R. C. Murphy, *Journal of the American Society for Mass Spectrometry*, 2007, **18**, 1646-1652.
92. E. B. Monroe, J. C. Jurchen, B. A. Koszczuk, J. L. Losh, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2006, **78**, 6826-6832.
93. K. R. Tucker, E. J. Lanni, L. A. Serebryannyy, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2011, **83**, 9181-9185.
94. N. Goto-Inoue, T. Hayasaka, N. Zaima, Y. Kashiwagi, M. Yamamoto, M. Nakamoto and M. Setou, *Journal of the American Society for Mass Spectrometry*, 2010, **21**, 1940-1943.
95. A. F. M. Altelaar, I. M. Taban, L. A. McDonnell, P. Verhaert, R. P. J. de Lange, R. A. H. Adan, W. J. Mooi, R. M. A. Heeren and S. R. Piersma, *International Journal of Mass Spectrometry*, 2007, **260**, 203-211.
96. S. Guenther, A. Rompp, W. Kummer and B. Spengler, *International Journal of Mass Spectrometry*, 2011, **305**, 228-237.
97. T. Harada, A. Yuba-Kubo, Y. Sugiura, N. Zaima, T. Hayasaka, N. Goto-Inoue, M. Wakui, M. Suematsu, K. Takeshita, K. Ogawa, Y. Yoshida and M. Setou, *Anal. Chem.*, 2009, **81**, 9153-9157.
98. M. C. Roy, H. Nakanishi, K. Takahashi, S. Nakanishi, S. Kajihara, T. Hayasaka, M. Setou, K. Ogawa, R. Taguchi and T. Naito, *Journal of Lipid Research*, 2011, **52**, 463-470.
99. B. Spengler and M. Hubert, *Journal of the American Society for Mass Spectrometry*, 2002, **13**, 735-748.
100. W. Bouschen, O. Schulz, D. Eikel and B. Spengler, *Rapid Communications in Mass Spectrometry*, 2010, **24**, 355-364.
101. P. L. Urban, T. Schmid, A. Amantonico and R. Zenobi, *Anal Chem*, 2011, **83**, 1843-1849.
102. B. Shrestha, J. M. Patt and A. Vertes, *Anal Chem*, 2011, **83**, 2947-2955.
103. Q. Zhao, C. H. Huang and F. Y. Li, *Chem. Soc. Rev.*, 2011, **40**, 2508-2524.
104. H. Mattoussi, G. Palui and H. B. Na, *Adv. Drug Deliv. Rev.*, 2012, **64**, 138-166.
105. P. Pierobon and G. Cappello, *Adv Drug Deliv Rev*, 2012, **64**, 167-178.
106. M.-H. Sung, *Cells*, 2013, **2**, 284-293.
107. S. Stehbens, H. Pemble, L. Murrow and T. Wittmann, *Methods in enzymology*, 2012, **504**, 293-313.
108. J. Kind, L. Pagie, H. Ortabozkoyun, S. Boyle, S. S. de Vries, H. Janssen, M. Amendola, L. D. Nolen, W. A. Bickmore and B. van Steensel, *Cell*, 2013, **153**, 178-192.
109. L. Lu, M. S. Ladinsky and T. Kirchhausen, *J Cell Biol*, 2011, **194**, 425-440.

110. A. K. Dunsch, D. Hammond, J. Lloyd, L. Schermelleh, U. Gruneberg and F. A. Barr, *J Cell Biol*, 2012, **198**, 1039-1054.
111. T. Ha, T. Enderle, D. F. Ogletree, D. S. Chemla, P. R. Selvin and S. Weiss, *Proc Natl Acad Sci U S A*, 1996, **93**, 6264-6268.
112. T. Forster, *Modern Quantum Chemistry*, Academic, New York, 1965.
113. S. Zadran, S. Standley, K. Wong, E. Otiniano, A. Amighi and M. Baudry, *Appl Microbiol Biotechnol*, 2012, **96**, 895-902.
114. J. Wiedenmann, F. Oswald and G. U. Nienhaus, *IUBMB Life*, 2009, **61**, 1029-1042.
115. T. Ansbacher, H. K. Srivastava, T. Stein, R. Baer, M. Merkx and A. Shurki, *Phys Chem Chem Phys*, 2012, **14**, 4109-4117.
116. B. R. Waterhouse, M. Gijsen, P. R. Barber, I. D. Tullis, B. Vojnovic and A. Kong, *Oncotarget*, 2011, **2**, 728-736.
117. M. Ouyang, H. Huang, N. C. Shaner, A. G. Remacle, S. A. Shiryaev, A. Y. Strongin, R. Y. Tsien and Y. Wang, *Cancer Res*, 2010, **70**, 2204-2212.
118. M. Willemse, E. Janssen, F. de Lange, B. Wieringa and J. Fransen, *Nat Biotechnol*, 2007, **25**, 170-172.
119. H. Imamura, K. P. Nhat, H. Togawa, K. Saito, R. Iino, Y. Kato-Yamada, T. Nagai and H. Noji, *Proc Natl Acad Sci U S A*, 2009, **106**, 15651-15656.
120. J. W. Borst, M. Willemse, R. Slijkhuis, G. van der Krogt, S. P. Laptenok, K. Jalink, B. Wieringa and J. A. Fransen, *PloS one*, 2010, **5**, e13862.
121. C. Depry, S. Mehta and J. Zhang, *Pflugers Arch*, 2013, **465**, 373-381.
122. D. Geissler, S. Stufler, H. G. Lohmannsroben and N. Hildebrandt, *J Am Chem Soc*, 2013, **135**, 1102-1109.
123. I. Ziomkiewicz, A. Loman, R. Klement, C. Fritsch, A. S. Klymchenko, G. Bunt, T. M. Jovin and D. J. Arndt-Jovin, *Cytometry A*, 2013.
124. W. Becker, *J. Microsc.*. 2012, **247**, 119-136.
125. Y. Sun, R. N. Day and A. Periasamy, *Nat Protoc*, 2011, **6**, 1324-1340.
126. E. A. Reits and J. J. Neefjes, *Nat Cell Biol*, 2001, **3**, E145-147.
127. F. Mueller, D. Mazza, T. J. Stasevich and J. G. McNally, *Curr Opin Cell Biol*, 2010, **22**, 403-411.
128. N. Rainy, D. Chetrit, V. Rouger, H. Vernitsky, O. Rechavi, D. Marguet, I. Goldstein, M. Ehrlich and Y. Kloog, *Cell Death Dis*, 2013, **4**, e726.
129. G. Herbomel, M. Kloster-Landsberg, E. G. Folco, E. Col, Y. Usson, C. Vourc'h, A. Delon and C. Souchier, *PloS one*, 2013, **8**, e67566.
130. K. B. Im, U. Schmidt, M. S. Kang, J. Y. Lee, F. Bestvater and M. Wachsmuth, *Cytometry A*, 2013.
131. E. L. Elson, *Biophysical journal*, 2011, **101**, 2855-2870.
132. P. Brazda, T. Szekeres, B. Bravics, K. Toth, G. Vamosi and L. Nagy, *Journal of cell science*, 2011, **124**, 3631-3642.
133. M. Dong, M. M. Martinez, M. F. Mayer and D. Pappas, *The Analyst*, 2012, **137**, 2997-3003.
134. M. M. Martinez, R. D. Reif and D. Pappas, *Anal Bioanal Chem*, 2010, **396**, 1177-1185.
135. H. Sadamoto, K. Saito, H. Muto, M. Kinjo and E. Ito, *PloS one*, 2011, **6**, e20285.
136. K. Saito, I. Wada, M. Tamura and M. Kinjo, *Biochemical and biophysical research communications*, 2004, **324**, 849-854.
137. A. Sasaki and M. Kinjo, *Journal of controlled release : official journal of the Controlled Release Society*, 2010, **143**, 104-111.
138. K. Bacia, S. A. Kim and P. Schwille, *Nat Methods*, 2006, **3**, 83-89.
139. G. Ball, R. M. Parton, R. S. Hamilton and I. Davis, *Methods in enzymology*, 2012, **504**, 29-55.

140. L. Schermelleh, R. Heintzmann and H. Leonhardt, *J Cell Biol*, 2010, **190**, 165-175.
141. D. C. Jans, C. A. Wurm, D. Riedel, D. Wenzel, F. Stagge, M. Deckers, P. Rehling and S. Jakobs, *Proc Natl Acad Sci U S A*, 2013, **110**, 8936-8941.
142. K. Ramser and D. Hanstorp, *J. Biophotonics*, 2010, **3**, 187-206.
143. F. Opazo, A. Punge, J. Buckers, P. Hoopmann, L. Kastrup, S. W. Hell and S. O. Rizzoli, *Traffic*, 2010, **11**, 800-812.
144. D. Wildanger, R. Medda, L. Kastrup and S. W. Hell, *J Microsc*, 2009, **236**, 35-43.
145. C. A. Wurm, D. Neumann, M. A. Lauterbach, B. Harke, A. Egner, S. W. Hell and S. Jakobs, *Proc Natl Acad Sci U S A*, 2011, **108**, 13546-13551.
146. J. Tonnesen and U. V. Nagerl, *Exp Neurol*, 2013, **242**, 33-40.
147. D. S. Lidke and K. A. Lidke, *Journal of cell science*, 2012, **125**, 2571-2580.
148. M. G. Gustafsson, *J Microsc*, 2000, **198**, 82-87.
149. M. G. L. Gustafsson, *Proc Natl Acad Sci U S A*, 2005, **102**, 13081-13086.
150. R. Heintzmann, T. M. Jovin and C. Cremer, *J. Opt. Soc. Am. A*, 2002, **19**, 1599-1609.
151. D. Baddeley, V. O. Chagin, L. Schermelleh, S. Martin, A. Pombo, P. M. Carlton, A. Gahl, P. Domaing, U. Birk, H. Leonhardt, C. Cremer and M. C. Cardoso, *Nucleic Acids Res*, 2010, **38**, e8.
152. L. Schermelleh, P. M. Carlton, S. Haase, L. Shao, L. Winoto, P. Kner, B. Burke, M. C. Cardoso, D. A. Agard, M. G. Gustafsson, H. Leonhardt and J. W. Sedat, *Science*, 2008, **320**, 1332-1336.
153. Y. Markaki, D. Smeets, S. Fiedler, V. J. Schmid, L. Schermelleh, T. Cremer and M. Cremer, *Bioessays*, 2012, **34**, 412-426.
154. K. A. Lidke, B. Rieger, T. M. Jovin and R. Heintzmann, *Opt Express*, 2005, **13**, 7052-7062.
155. P. Sengupta, S. Van Engelenburg and J. Lippincott-Schwartz, *Developmental cell*, 2012, **23**, 1092-1102.
156. T. Dertinger, R. Colyer, R. Vogel, J. Enderlein and S. Weiss, *Opt Express*, 2010, **18**, 18875-18885.
157. P. Dedecker, G. C. H. Mo, T. Dertinger and J. Zhang, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 10909-10914.
158. S. Cox, E. Rosten, J. Monypenny, T. Jovanovic-Talisman, D. T. Burnette, J. Lippincott-Schwartz, G. E. Jones and R. Heintzmann, *Nat. Methods*, 2012, **9**, 195-200.
159. J. W. Uhr, M. L. Huebschman, E. P. Frenkel, N. L. Lane, R. Ashfaq, H. Liu, D. R. Rana, L. Cheng, A. T. Lin, G. A. Hughes, X. J. Zhang and H. R. Garner, *Transl Res*, 2012, **159**, 366-375.
160. L. Gao, R. T. Kester, N. Hagen and T. S. Tkaczyk, *Opt Express*, 2010, **18**, 14330-14344.
161. A. D. Elliott, L. Gao, A. Ustione, N. Bedard, R. Kester, D. W. Piston and T. S. Tkaczyk, *Journal of cell science*, 2012, **125**, 4833-4840.
162. S. J. Leavesley, N. Annamdevula, J. Boni, S. Stocker, K. Grant, B. Troyanovsky, T. C. Rich and D. F. Alvarez, *J Biophotonics*, 2012, **5**, 67-84.
163. M. Coelho, N. Maghelli and I. M. Tolic-Norrelykke, *Integr. Biol.*, 2013, **5**, 748-758.
164. R. Iino, I. Koyama and A. Kusumi, *Biophysical journal*, 2001, **80**, 2667-2677.
165. K. Gowrishankar, S. Ghosh, S. Saha, R. C, S. Mayor and M. Rao, *Cell*, 2012, **149**, 1353-1367.
166. K. G. Stenkula, V. A. Lizunov, S. W. Cushman and J. Zimmerberg, *Cell metabolism*, 2010, **12**, 250-259.
167. L. Wang, M. A. Bittner, D. Axelrod and R. W. Holz, *Molecular biology of the cell*, 2008, **19**, 3944-3955.
168. Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii and M. Hide, *Allergol. Int.*, 2013, **62**, 163-169.

169. D. Peelen, V. Kodoyianni, J. Lee, T. Zheng, M. R. Shortreed and L. M. Smith, *Journal of proteome research*, 2006, **5**, 1580-1585.
170. Y. Yanase, T. Hiragun, S. Kaneko, H. J. Gould, M. W. Greaves and M. Hide, *Biosens Bioelectron*, 2010, **26**, 674-681.
171. M. Horii, H. Shinohara, Y. Iribe and M. Suzuki, *The Analyst*, 2011, **136**, 2706-2711.
172. M. West, A. T. Ellis, P. J. Potts, C. Streli, C. Vanhoof, D. Wegrzynek and P. Wobrauschek, *J. Anal. At. Spectrom.*, 2011, **26**, 1919-1963.
173. R. Ortega, *J. Anal. At. Spectrom.*, 2011, **26**, 23-29.
174. C. T. Dillon, *Aust. J. Chem.*, 2012, **65**, 204-217.
175. W. Meyer-Ilse, D. Hamamoto, A. Nair, S. A. Lelievre, G. Denbeaux, L. Johnson, A. L. Pearson, D. Yager, M. A. Legros and C. A. Larabell, *J Microsc*, 2001, **201**, 395-403.
176. S. Ahn, S. Y. Jung and S. J. Lee, *Molecules*, 2013, **18**, 5858-5890.
177. H. N. Chapman and K. A. Nugent, *Nat. Photonics*, 2010, **4**, 833-839.
178. G. McDermott, M. A. Le Gros and C. A. Larabell, in *Annual Review of Physical Chemistry, Vol 63*, eds. M. A. Johnson and T. J. Martinez, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 63, pp. 225-239.
179. G. E. Murphy, K. Narayan, B. C. Lowekamp, L. M. Hartnell, J. A. Heymann, J. Fu and S. Subramaniam, *J Struct Biol*, 2011, **176**, 268-278.
180. Z. Li, L. Q. Chu, J. V. Sweedler and P. W. Bohn, *Anal. Chem.*, 2010, **82**, 2608-2611.
181. S. Yilmaz and A. K. Singh, *Curr. Opin. Biotechnol.*, 2012, **23**, 437-443.
182. R. S. Lasken, *Nat Rev Microbiol*, 2007, **10**, 631-640.
183. H. C. Fan, J. B. Wang, A. Potanina and S. R. Quake, *Nat. Biotechnol.*, 2011, **29**, 51-+.
184. N. Navin, J. Kendall, J. Troge, P. Andrews, L. Rodgers, J. McIndoo, K. Cook, A. Stepansky, D. Levy, D. Esposito, L. Muthuswamy, A. Krasnitz, W. R. McCombie, J. Hicks and M. Wigler, *Nature*, 2011, **472**, 90-U119.
185. M. Malecki and W. Szybalski, *Gene*, 2012, **493**, 132-139.
186. Y. Hou, L. T. Song, P. Zhu, B. Zhang, Y. Tao, X. Xu, F. Q. Li, K. Wu, J. Liang, D. Shao, H. J. Wu, X. F. Ye, C. Ye, R. H. Wu, M. Jian, Y. Chen, W. Xie, R. R. Zhang, L. Chen, X. Liu, X. T. Yao, H. C. Zheng, C. Yu, Q. B. Li, Z. L. Gong, M. Mao, X. Yang, L. Yang, J. X. Li, W. Wang, Z. H. Lu, N. Gu, G. Laurie, L. Bolund, K. Kristiansen, J. Wang, H. M. Yang, Y. R. Li, X. Q. Zhang and J. Wang, *Cell*, 2012, **148**, 873-885.
187. X. Xu, Y. Hou, X. Yin, L. Bao, A. Tang, L. Song, F. Li, S. Tsang, K. Wu, H. Wu, W. He, L. Zeng, M. Xing, R. Wu, H. Jiang, X. Liu, D. Cao, G. Guo, X. Hu, Y. Gui, Z. Li, W. Xie, X. Sun, M. Shi, Z. Cai, B. Wang, M. Zhong, J. Li, Z. Lu, N. Gu, X. Zhang, L. Goodman, L. Bolund, J. Wang, H. Yang, K. Kristiansen, M. Dean, Y. Li and J. Wang, *Cell*, 2012, **148**, 886-895.
188. D. Wang and S. Bodovitz, *Trends Biotechnol*, 2010, **28**, 281-290.
189. J. Tischler and M. A. Surani, *Curr. Opin. Biotechnol.*, 2013, **24**, 69-78.
190. A. Stahlberg and M. Bengtsson, *Methods*, 2010, **50**, 282-288.
191. A. Raj and A. van Oudenaarden, *Cell*, 2008, **135**, 216-226.
192. M. B. Elowitz, A. J. Levine, E. D. Siggia and P. S. Swain, *Science*, 2002, **297**, 1183-1186.
193. J. M. Raser and E. K. O'Shea, *Science*, 2004, **304**, 1811-1814.
194. A. Raj, C. S. Peskin, D. Tranchina, D. Y. Vargas and S. Tyagi, *PLoS biology*, 2006, **4**, e309.
195. A. Stahlberg, M. Kubista and P. Aman, *Expert Review of Molecular Diagnostics*, 2011, **11**, 735-740.
196. J. Eberwine, D. Lovatt, P. Buckley, H. Dueck, C. Francis, T. K. Kim, J. Lee, M. Lee, K. Miyashiro, J. Morris, T. Peritz, T. Schochet, J. Spaethling, J. Y. Sul and J. Kim, *J. R. Soc. Interface*, 2012, **9**, 3165-3183.

197. S. A. Bustin, V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer, *Clin Chem*, 2009, **55**, 611-622.
198. K. A. Heyries, C. Tropini, M. VanInsberghe, C. Doolin, O. I. Petriv, A. Singhal, K. Leung, C. B. Hughesman and C. L. Hansen, *Nat. Methods*, 2011, **8**, 649-U664.
199. J. B. Fan, J. Chen, C. S. April, J. S. Fisher, B. Klotzle, M. Bibikova, F. Kaper, M. Ronaghi, S. Linnarsson, T. Ota, J. Chien, L. C. Laurent, J. F. Loring, S. V. Nisperos, G. Y. Chen and J. F. Zhong, *PloS one*, 2012, **7**, e30794.
200. J. M. Levsky and R. H. Singer, *Journal of cell science*, 2003, **116**, 2833-2838.
201. P. Lichter, T. Cremer, J. Borden, L. Manuelidis and D. C. Ward, *Hum Genet*, 1988, **80**, 224-234.
202. L. Chatre and M. Ricchetti, *Journal of cell science*, 2013, **126**, 914-926.
203. A. M. Femino, F. S. Fay, K. Fogarty and R. H. Singer, *Science*, 1998, **280**, 585-590.
204. S. Itzkovitz and A. van Oudenaarden, *Nat Methods*, 2011, **8**, S12-19.
205. A. Raj, P. van den Bogaard, S. A. Rifkin, A. van Oudenaarden and S. Tyagi, *Nat Methods*, 2008, **5**, 877-879.
206. Y. Buganim, D. A. Faddah, A. W. Cheng, E. Itskovich, S. Markoulaki, K. Ganz, S. L. Klemm, A. van Oudenaarden and R. Jaenisch, *Cell*, 2012, **150**, 1209-1222.
207. S. Itzkovitz, A. Lyubimova, I. C. Blat, M. Maynard, J. van Es, J. Lees, T. Jacks, H. Clevers and A. van Oudenaarden, *Nat Cell Biol*, 2012, **14**, 106-114.
208. J. Lu and A. Tsourkas, *Nucleic Acids Res*, 2009, **37**, e100.
209. C. Larsson, I. Grundberg, O. Soderberg and M. Nilsson, *Nat Methods*, 2010, **7**, 395-397.
210. N. Rosenfeld, J. W. Young, U. Alon, P. S. Swain and M. B. Elowitz, *Science*, 2005, **307**, 1962-1965.
211. J. Selimkhanov, J. Hasty and L. S. Tsimring, *Curr Opin Biotechnol*, 2012, **23**, 34-40.
212. D. Bakstad, A. Adamson, D. G. Spiller and M. R. White, *Curr Opin Biotechnol*, 2012, **23**, 103-109.
213. E. Bertrand, P. Chartrand, M. Schaefer, S. M. Shenoy, R. H. Singer and R. M. Long, *Mol. Cell.*, 1998, **2**, 437-445.
214. S. Tyagi and F. R. Kramer, *Nat. Biotechnol.*, 1996, **14**, 303-308.
215. J. M. Spaethling and J. H. Eberwine, *Current opinion in pharmacology*, 2013.
216. T. Bartfai, P. T. Buckley and J. Eberwine, *Trends in pharmacological sciences*, 2012, **33**, 9-16.
217. F. Tang, C. Barbacioru, Y. Wang, E. Nordman, C. Lee, N. Xu, X. Wang, J. Bodeau, B. B. Tuch, A. Siddiqui, K. Lao and M. A. Surani, *Nat Methods*, 2009, **6**, 377-382.
218. T. Kivioja, A. Vaharautio, K. Karlsson, M. Bonke, M. Enge, S. Linnarsson and J. Taipale, *Nat. Methods*, 2012, **9**, 72-U183.
219. Q. F. Wills, K. J. Livak, A. J. Tipping, T. Enver, A. J. Goldson, D. W. Sexton and C. Holmes, *Nat. Biotechnol.*, 2013, **31**, 748-+.
220. M. J. Levesque, P. Ginart, Y. C. Wei and A. Raj, *Nat. Methods*, 2013, **10**, 865-+.
221. M. Wu and A. K. Singh, *Curr Opin Biotechnol*, 2012, **23**, 83-88.
222. A. F. M. Altelaar and A. J. R. Heck, *Current Opinion in Chemical Biology*, 2012, **16**, 206-213.
223. K. He, N. Wang, W. H. Li and X. M. Zhang, *Curr. Opin. Biotechnol.*, 2012, **23**, 120-125.
224. S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2008, **80**, 7128-7136.
225. P. L. Urban, K. Jefimovs, A. Amantonico, S. R. Fagerer, T. Schmid, S. Madler, J. Puigmarti-Luis, N. Goedecke and R. Zenobi, *Lab Chip*, 2010, **10**, 3206-3209.
226. J. S. Mellors, K. Jorabchi, L. M. Smith and J. M. Ramsey, *Anal. Chem.*, 2010, **82**, 967-973.

227. B. Huang, H. K. Wu, D. Bhaya, A. Grossman, S. Granier, B. K. Kobilka and R. N. Zare, *Science*, 2007, **315**, 81-84.
228. Q. H. Shi, L. D. Qin, W. Wei, F. Geng, R. Fan, Y. S. Shin, D. L. Guo, L. Hood, P. S. Mischel and J. R. Heath, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 419-424.
229. C. Ma, R. Fan, H. Ahmad, Q. Shi, B. Comin-Anduix, T. Chodon, R. C. Koya, C. C. Liu, G. A. Kwong, C. G. Radu, A. Ribas and J. R. Heath, *Nature medicine*, 2011, **17**, 738-743.
230. C. Ma, R. Fan and M. Elitas, *Frontiers in oncology*, 2013, **3**, 133.
231. J. Choi, K. R. Love, Y. Gong, T. M. Gierahn and J. C. Love, *Anal. Chem.*, 2011, **83**, 6890-6895.
232. A. Salehi-Reyhani, J. Kaplinsky, E. Burgin, M. Novakova, A. J. Demello, R. H. Templer, P. Parker, M. A. A. Neil, O. Ces, P. French, K. R. Willison and D. Klug, *Lab Chip*, 2011, **11**, 1256-1261.
233. A. Salehi-Reyhani, S. Sharma, E. Burgin, M. Barclay, A. Cass, M. A. A. Neil, O. Ces, K. R. Willison and D. R. Klug, *Lab Chip*, 2013, **13**, 2066-2074.
234. A. Amantonico, P. L. Urban and R. Zenobi, *Anal. Bioanal. Chem.*, 2010, **398**, 2493-2504.
235. A. Amantonico, P. L. Urban, S. R. Fagerer, R. M. Balabin and R. Zenobi, *Anal Chem*, 2010, **82**, 7394-7400.
236. E. P. Go, *Journal of neuroimmune pharmacology : the official journal of the Society on NeuroImmune Pharmacology*, 2010, **5**, 18-30.
237. S. S. Rubakhin, E. J. Lanni and J. V. Sweedler, *Curr. Opin. Biotechnol.*, 2013, **24**, 95-104.
238. A. Amantonico, J. Y. Oh, J. Sobek, M. Heinemann and R. Zenobi, *Angew. Chem.-Int. Edit.*, 2008, **47**, 5382-5385.
239. M. Heinemann and R. Zenobi, *Curr Opin Biotechnol*, 2011, **22**, 26-31.
240. P. Nemes, A. M. Knolhoff, S. S. Rubakhin and J. V. Sweedler, *Anal. Chem.*, 2011, **83**, 6810-6817.
241. P. Nemes, S. S. Rubakhin, J. T. Aerts and J. V. Sweedler, *Nat. Protoc.*, 2013, **8**, 783-799.
242. M. Lorenzo Tejedor, H. Mizuno, N. Tsuyama, T. Harada and T. Masujima, *Anal Chem*, 2012, **84**, 5221-5228.
243. T. Okajima, *Curr. Pharm. Biotechnol.*, 2012, **13**, 2623-2631.
244. K. Kleparnik, *Electrophoresis*, 2013, **34**, 70-85.
245. O. Ornatsky, D. Bandura, V. Baranov, M. Nitz, M. A. Winnik and S. Tanner, *J. Immunol. Methods*, 2010, **361**, 1-20.
246. S. C. Bendall, E. F. Simonds, P. Qiu, E. A. D. Amir, P. O. Krutzik, R. Finck, R. V. Bruggner, R. Melamed, A. Trejo, O. I. Ornatsky, R. S. Balderas, S. K. Plevritis, K. Sachs, D. Pe'er, S. D. Tanner and G. P. Nolan, *Science*, 2011, **332**, 687-696.
247. C. N. Tsang, K. S. Ho, H. Z. Sun and W. T. Chan, *Journal of the American Chemical Society*, 2011, **133**, 7355-7357.
248. Y. Gao, Y. M. Lin, B. C. Zhang, D. X. Zou, M. H. He, B. Dong, W. Hang and B. L. Huang, *Anal. Chem.*, 2013, **85**, 4268-4272.
249. A. Motta, D. Paris and D. Melck, *Anal. Chem.*, 2010, **82**, 2405-2411.
250. A. H. Zhang, H. Sun, S. Qiu and X. J. Wang, *Magn. Reson. Chem.*, 2013, **51**, 549-556.
251. T. Gebregiworgis and R. Powers, *Comb. Chem. High Throughput Screen*, 2012, **15**, 595-610.
252. S. C. Grant, N. R. Aiken, H. D. Plant, S. Gibbs, T. H. Mareci, A. G. Webb and S. J. Blackband, *Magnetic resonance in medicine : official journal of the Society of Magnetic Resonance in Medicine / Society of Magnetic Resonance in Medicine*, 2000, **44**, 19-22.

253. S. C. Lee, J. H. Cho, D. Mietchen, Y. S. Kim, K. S. Hong, C. Lee, D. Kang, K. D. Park, B. S. Choi and C. Cheong, *Biophysical journal*, 2006, **90**, 1797-1803.
254. X. Y. Ye, S. S. Rubakhin and J. V. Sweedler, *The Analyst*, 2008, **133**, 423-433.
255. Y. Zheng, J. Nguyen, Y. Wei and Y. Sun, *Lab Chip*, 2013, **13**, 2464-2483.
256. H. Morgan, T. Sun, D. Holmes, S. Gawad and N. G. Green, *J. Phys. D-Appl. Phys.*, 2007, **40**, 61-70.
257. B. J. Kirby, *Micro- and Nanoscale Fluid Mechanics - Transport in Microfluidic Devices*, Cambridge University Press, New York, 2010.
258. T. Sun and H. Morgan, *Microfluid. Nanofluid.*, 2010, **8**, 423-443.
259. K. C. Cheung, M. Di Berardino, G. Schade-Kampmann, M. Hebeisen, A. Pierzchalski, J. Bocsi, A. Mittag and A. Tarnok, *Cytometry Part A*, 2010, **77A**, 648-666.
260. Y. Zheng, E. Shojaei-Baghini, C. Wang and Y. Sun, *Biosens. Bioelectron.*, 2013, **42**, 496-502.
261. X. J. Han, C. van Berkel, J. Gwyer, L. Capretto and H. Morgan, *Anal. Chem.*, 2012, **84**, 1070-1075.
262. C. van Berkel, J. D. Gwyer, S. Deane, N. Green, J. Holloway, V. Hollis and H. Morgan, *Lab Chip*, 2011, **11**, 1249-1255.
263. D. Holmes, D. Pettigrew, C. H. Reccius, J. D. Gwyer, C. van Berkel, J. Holloway, D. E. Davies and H. Morgan, *Lab Chip*, 2009, **9**, 2881-2889.
264. S. B. Cho and H. Thielecke, *Biosens. Bioelectron.*, 2007, **22**, 1764-1768.
265. S. Cho, M. Castellarnau, J. Samitier and H. Thielecke, *Sensors*, 2008, **8**, 1198-1211.
266. C. V. Kurz, H. Buth, A. Sossalla, V. Vermeersch, V. Toncheva, P. Dubruel, E. Schacht and H. Thielecke, *Biosens. Bioelectron.*, 2011, **26**, 3405-3412.
267. D. Mondal, C. Roychaudhuri, L. Das and J. Chatterjee, *Biomed Microdevices*, 2012, **14**, 955-964.
268. S. K. Lee, W. F. Boron and M. D. Parker, *Sensors*, 2013, **13**, 984-1003.
269. M. Lindau, *Biochim. Biophys. Acta-Gen. Subj.*, 2012, **1820**, 1234-1242.
270. G. Minetti, S. Egee, D. Morsdorf, P. Steffen, A. Makhro, C. Achilli, A. Ciana, J. Wang, G. Bouyer, I. Bernhardt, C. Wagner, S. Thomas, A. Bogdanova and L. Kaestner, *Blood Rev.*, 2013, **27**, 91-101.
271. M. Bebarova, *Gen. Physiol. Biophys.*, 2012, **31**, 131-140.
272. C. Grewer, A. Gameiro, T. Mager and K. Fendler, in *Annual Review of Biophysics, Vol 42*, Annual Reviews, Palo Alto, Editon edn., 2013, vol. 42, pp. 95-120.
273. L. Yobas, *J. Micromech. Microeng.*, 2013, **23**, 16.
274. G. S. Wilson and M. A. Johnson, *Chem. Rev.*, 2008, **108**, 2462-2481.
275. B. Reid and M. Zhao, *Commun Integr Biol*, 2011, **4**, 524-527.
276. E. S. McLamore and D. M. Porterfield, *Chem. Soc. Rev.*, 2011, **40**, 5308-5320.
277. D. M. Porterfield, *Biosens. Bioelectron.*, 2007, **22**, 1186-1196.
278. R. I. Dmitriev and D. B. Papkovsky, *Cell. Mol. Life Sci.*, 2012, **69**, 2025-2039.
279. C. F. Wu, B. Bull, K. Christensen and J. McNeill, *Angew. Chem.-Int. Edit.*, 2009, **48**, 2741-2745.
280. M. P. Coogan, J. B. Court, V. L. Gray, A. J. Hayes, S. H. Lloyd, C. O. Millet, S. J. A. Pope and D. Lloyd, *Photochem. Photobiol. Sci.*, 2010, **9**, 103-109.
281. T. C. O'Riordan, A. V. Zhdanov, G. V. Ponomarev and D. B. Papkovsky, *Anal. Chem.*, 2007, **79**, 9414-9419.
282. U. Neugebauer, Y. Pellegrin, M. Devocelle, R. J. Forster, W. Signac, N. Morand and T. E. Keyes, *Chemical Communications*, 2008, 5307-5309.
283. R. I. Dmitriev, A. V. Zhdanov, G. V. Ponomarev, D. V. Yashunski and D. B. Papkovsky, *Anal. Biochem.*, 2010, **398**, 24-33.
284. R. Weigert, M. Sramkova, L. Parente, P. Amornphimoltham and A. Masedunskas, *Histochem Cell Biol*, 2010, **133**, 481-491.

285. T. Terai and T. Nagano, *Pflugers Arch*, 2013, **465**, 347-359.
286. C. Grienberger and A. Konnerth, *Neuron*, 2012, **73**, 862-885.
287. P. Kettunen, *Adv Exp Med Biol*, 2012, **740**, 1039-1071.
288. T. Riemensperger, U. Pech, S. Dipt and A. Fiala, *Biochim Biophys Acta*, 2012, **1820**, 1169-1178.
289. K. K. W. Lo, K. Y. Zhang and S. P. Y. Li, *Eur. J. Inorg. Chem.*, 2011, 3551-3568.
290. M. R. Gill and J. A. Thomas, *Chem. Soc. Rev.*, 2012, **41**, 3179-3192.
291. J. J. Shin and C. J. Loewen, *BMC Biol*, 2011, **9**, 85.
292. J. R. Casey, S. Grinstein and J. Orlowski, *Nat Rev Mol Cell Biol*, 2010, **11**, 50-61.
293. J. Llopis, J. M. McCaffery, A. Miyawaki, M. G. Farquhar and R. Y. Tsien, *Proc Natl Acad Sci U S A*, 1998, **95**, 6803-6808.
294. M. Tantama, Y. P. Hung and G. Yellen, *J Am Chem Soc*, 2011, **133**, 10034-10037.
295. J. Kneipp, H. Kneipp, B. Wittig and K. Kneipp, *J. Phys. Chem. C*, 2010, **114**, 7421-7426.
296. R. Barer, *Nature*, 1953, **172**, 1097-1098.
297. N. Lue, W. Choi, G. Popescu, Z. Yaqoob, K. Badizadegan, R. R. Dasari and M. S. Feld, *J Phys Chem A*, 2009, **113**, 13327-13330.
298. W. Choi, C. Fang-Yen, K. Badizadegan, S. Oh, N. Lue, R. R. Dasari and M. S. Feld, *Nat Methods*, 2007, **4**, 717-719.
299. Y. Sung, W. Choi, N. Lue, R. R. Dasari and Z. Yaqoob, *PloS one*, 2012, **7**, e49502.
300. J. Reed, J. Chun, T. A. Zangle, S. Kalim, J. S. Hong, S. E. Pefley, X. Zheng, J. K. Gimzewski and M. A. Teitell, *Biophysical journal*, 2011, **101**, 1025-1031.
301. M. Mir, Z. Wang, Z. Shen, M. Bednarz, R. Bashir, I. Golding, S. G. Prasanth and G. Popescu, *Proc Natl Acad Sci U S A*, 2011, **108**, 13124-13129.
302. Z. Wang, L. Millet, M. Mir, H. Ding, S. Unarunotai, J. Rogers, M. U. Gillette and G. Popescu, *Opt Express*, 2011, **19**, 1016-1026.
303. T. P. Burg, M. Godin, S. M. Knudsen, W. Shen, G. Carlson, J. S. Foster, K. Babcock and S. R. Manalis, *Nature*, 2007, **446**, 1066-1069.
304. M. Godin, F. F. Delgado, S. M. Son, W. H. Grover, A. K. Bryan, A. Tzur, P. Jorgensen, K. Payer, A. D. Grossman, M. W. Kirschner and S. R. Manalis, *Nat. Methods*, 2010, **7**, 387-U370.
305. J. J. Li and K. D. Zhu, *Physics Reports-Review Section of Physics Letters*, 2013, **525**, 223-254.
306. W. H. Grover, A. K. Bryan, M. Diez-Silva, S. Suresh, J. M. Higgins and S. R. Manalis, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 10992-10996.
307. D. Kirmizis and S. Logothetidis, *Int. J. Nanomed.*, 2010, **5**, 137-145.
308. Y. J. Li, C. Wen, H. M. Xie, A. P. Ye and Y. J. Yin, *Colloid Surf. B-Biointerfaces*, 2009, **70**, 169-173.
309. C. T. Lim, E. H. Zhou and S. T. Quek, *J. Biomech.*, 2006, **39**, 195-216.
310. E. H. Zhou, S. T. Quek and C. T. Lim, *Biomech. Model. Mechanobiol.*, 2010, **9**, 563-572.
311. D. H. Kim, P. K. Wong, J. Park, A. Levchenko and Y. Sun, in *Annual Review of Biomedical Engineering*, Annual Reviews, Palo Alto, Editon edn., 2009, vol. 11, pp. 203-233.
312. O. Loh, A. Vaziri and H. Espinosa, *Exp. Mech.*, 2009, **49**, 105-124.
313. M. Lekka, K. Pogoda, J. Gostek, O. Klymenko, S. Prauzner-Bechcicki, J. Wiltowska-Zuber, J. Jaczewska, J. Lekki and Z. Stachura, *Micron*, 2012, **43**, 1259-1266.
314. I. Sraj, C. D. Eggleton, R. Jimenez, E. Hoover, J. Squier, J. Chichester and D. W. M. Marr, *J. Biomed. Opt.*, 2010, **15**.
315. P. C. Ashok and K. Dholakia, *Curr Opin Biotechnol*, 2012, **23**, 16-21.

316. J. M. A. Mauritz, T. Tiffert, R. Seear, F. Lautenschlager, A. Esposito, V. L. Lew, J. Guck and C. F. Kaminski, *J. Biomed. Opt.*, 2010, **15**.
317. D. R. Gossett, H. T. K. Tse, S. A. Lee, Y. Ying, A. G. Lindgren, O. O. Yang, J. Y. Rao, A. T. Clark and D. Di Carlo, *Proc. Natl. Acad. Sci. U. S. A.*, 2012, **109**, 7630-7635.
318. D. Di Carlo, *Jala*, 2012, **17**, 32-42.
319. Y. Zheng and Y. Sun, *Micro & Nano Letters*, 2011, **6**, 327-331.
320. G. S. Du, A. Ravetto, Q. Fang and J. M. J. den Toonder, *Biomedical Microdevices*, 2011, **13**, 29-40.
321. Y. C. Kim, S. J. Park and J. K. Park, *The Analyst*, 2008, **133**, 1432-1439.
322. F. A. Carvalho and N. C. Santos, *IUBMB Life*, 2012, **64**, 465-472.
323. V. Fernandez-Duenas, J. Llorente, J. Gandia, D. O. Borroto-Escuela, L. F. Agnati, C. I. Tasca, K. Fuxe and F. Ciruela, *Methods*, 2012, **57**, 467-472.
324. D. G. Spiller, C. D. Wood, D. A. Rand and M. R. White, *Nature*, 2010, **465**, 736-745.
325. J. Huang, C. Meyer and C. Zhu, *Mol Immunol*, 2012, **52**, 155-164.
326. B. Koos, L. Andersson, C. M. Clausson, K. Grannas, A. Klaesson, G. Cane and O. Soderberg, *Curr Top Microbiol Immunol*, 2013.
327. O. Soderberg, M. Gullberg, M. Jarvius, K. Ridderstrale, K. J. Leuchowius, J. Jarvius, K. Wester, P. Hydbring, F. Bahram, L. G. Larsson and U. Landegren, *Nat Methods*, 2006, **3**, 995-1000.
328. O. Soderberg, K. J. Leuchowius, M. Gullberg, M. Jarvius, I. Weibrecht, L. G. Larsson and U. Landegren, *Methods*, 2008, **45**, 227-232.
329. M. Blazek, C. Betz, M. Reth, M. N. Hall, R. Zengerle and M. Meier, *Mol Cell Proteomics*, 2013.
330. Y. Yanase, T. Hiragun, T. Yanase, T. Kawaguchi, K. Ishii and M. Hide, *Allergology international : official journal of the Japanese Society of Allergology*, 2013, **62**, 163-169.
331. D. H. M. Dam, J. H. Lee, P. N. Sisco, D. T. Co, M. Zhang, M. R. Wasielewski and T. W. Odom, *ACS Nano*, 2012, **6**, 3318-3326.
332. Z. Feng, W. Zhang, J. Xu, C. Gauron, B. Ducos, S. Vriz, M. Volovitch, L. Jullien, S. Weiss and D. Bensimon, *Rep Prog Phys*, 2013, **76**, 072601.
333. D. Oron, E. Papagiakoumou, F. Anselmi and V. Emiliani, in *Optogenetics: Tools for Controlling and Monitoring Neuronal Activity*, Elsevier Science Bv, Amsterdam, Editon edn., 2012, vol. 196, pp. 119-143.
334. R. H. W. Lam, Y. B. Sun, W. Q. Chen and J. P. Fu, *Lab Chip*, 2012, **12**, 1865-1873.
335. N. J. Sniadecki, A. Anguelouch, M. T. Yang, C. M. Lamb, Z. Liu, S. B. Kirschner, Y. Liu, D. H. Reich and C. S. Chen, *Proc Natl Acad Sci U S A*, 2007, **104**, 14553-14558.
336. S. K. Yoo, P. Y. Lam, M. R. Eichelberg, L. Zasadil, W. M. Bement and A. Huttenlocher, *Journal of cell science*, 2012, **125**, 5702-5710.
337. X. Liu, F. Long, H. Peng, S. J. Aerni, M. Jiang, A. Sanchez-Blanco, J. I. Murray, E. Preston, B. Mericle, S. Batzoglou, E. W. Myers and S. K. Kim, *Cell*, 2009, **139**, 623-633.
338. I. Topalidou, A. van Oudenaarden and M. Chalfie, *Proc Natl Acad Sci U S A*, 2011, **108**, 4063-4068.
339. M. Weber and J. Huisken, *Curr Opin Genet Dev*, 2011, **21**, 566-572.
340. Y. Wu, A. Ghitani, R. Christensen, A. Santella, Z. Du, G. Rondeau, Z. Bao, D. Colon-Ramos and H. Shroff, *Proc Natl Acad Sci U S A*, 2011, **108**, 17708-17713.
341. U. Krzic, S. Gunther, T. E. Saunders, S. J. Streichan and L. Hufnagel, *Nat Methods*, 2012, **9**, 730-733.
342. L. Gao, L. Shao, C. D. Higgins, J. S. Poulton, M. Peifer, M. W. Davidson, X. Wu, B. Goldstein and E. Betzig, *Cell*, 2012, **151**, 1370-1385.

343. F. Progatzky, M. J. Dallman and C. Lo Celso, *Interface focus*, 2013, **3**, 20130001.
344. W. Denk, J. H. Strickler and W. W. Webb, *Science*, 1990, **248**, 73-76.
345. N. R. Bhakta and R. S. Lewis, *Seminars in immunology*, 2005, **17**, 411-420.
346. M. D. Cahalan, I. Parker, S. H. Wei and M. J. Miller, *Nature reviews. Immunology*, 2002, **2**, 872-880.
347. M. Mues, I. Bartholomaus, T. Thestrup, O. Griesbeck, H. Wekerle, N. Kawakami and G. Krishnamoorthy, *Nature medicine*, 2013, **19**, 778-783.
348. L. V. Wang, *Nat Photonics*, 2009, **3**, 503-509.
349. L. Wang, K. Maslov and L. V. Wang, *Proc Natl Acad Sci U S A*, 2013, **110**, 5759-5764.
350. C. Q. Yi, C. W. Li, S. L. Ji and M. S. Yang, *Anal. Chim. Acta*, 2006, **560**, 1-23.
351. S. Lindstrom and H. Andersson-Svahn, *Lab Chip*, 2010, **10**, 3363-3372.
352. J. R. Moffitt, Y. R. Chemla, S. B. Smith and C. Bustamante, in *Annual Review of Biochemistry*, Annual Reviews, Palo Alto, Editon edn., 2008, vol. 77, pp. 205-228.
353. H. Zhang and K. K. Liu, *J. R. Soc. Interface*, 2008, **5**, 671-690.
354. U. Neugebauer, T. Bocklitz, J. H. Clement, C. Krafft and J. Popp, *The Analyst*, 2010, **135**, 3178-3182.
355. B. Kemper, P. Langehanenberg, A. Hoink, G. von Bally, F. Wottowah, S. Schinkinger, J. Guck, J. Kas, I. Bredebusch, J. Schnekenburger and K. Schutze, *J. Biophotonics*, 2010, **3**, 425-431.
356. M. Gyger, D. Rose, R. Stange, T. Kiessling, M. Zink, B. Fabry and J. A. Kas, *Opt. Express*, 2011, **19**, 19212-19222.
357. A. Farre, C. Lopez-Quesada, J. Andilla, E. Martin-Badosa and M. Montes-Usategui, *Opt. Eng.*, 2010, **49**, 6.
358. E. D'Este, G. Baj, P. Beuzer, E. Ferrari, G. Pinato, E. Tongiorgi and D. Cojoc, *Integr. Biol.*, 2011, **3**, 568-577.
359. L. B. Oddershede, *Nat. Chem. Biol.*, 2012, **8**, 879-886.
360. H. M. K. Wong, M. Righini, J. C. Gates, P. G. R. Smith, V. Pruneri and R. Quidant, *Appl. Phys. Lett.*, 2011, **99**, 3.
361. M. L. Juan, M. Righini and R. Quidant, *Nat. Photonics*, 2011, **5**, 349-356.
362. M. Righini, G. Volpe, C. Girard, D. Petrov and R. Quidant, *Phys. Rev. Lett.*, 2008, **100**.
363. A. N. Grigorenko, N. W. Roberts, M. R. Dickinson and Y. Zhang, *Nat. Photonics*, 2008, **2**, 365-370.
364. A. E. Christakou, M. Ohlin, B. Vanherberghen, M. A. Khorshidi, N. Kadri, T. Frisk, M. Wiklund and B. Onfelt, *Integr Biol (Camb)*, 2013, **5**, 712-719.
365. B. Vanherberghen, O. Manneberg, A. Christakou, T. Frisk, M. Ohlin, H. M. Hertz, B. Onfelt and M. Wiklund, *Lab Chip*, 2010, **10**, 2727-2732.
366. M. Ohlin, A. E. Christakou, T. Frisk, B. Onfelt and M. Wiklund, *J. Micromech. Microeng.*, 2013, **23**.
367. I. De Vlaminck and C. Dekker, in *Annual Review of Biophysics, Vol 41*, ed. D. C. Rees, Annual Reviews, Palo Alto, Editon edn., 2012, vol. 41, pp. 453-472.
368. T. Shimada, W. Watanabe, S. Matsunaga, T. Higashi, H. Ishii, K. Fukui, K. Isobe and K. Itoh, *Opt Express*, 2005, **13**, 9869-9880.
369. R. B. Brown and J. Audet, *J. R. Soc. Interface*, 2008, **5**, S131-S138.
370. Y. Q. Lin, R. Trouillon, G. Safina and A. G. Ewing, *Anal. Chem.*, 2011, **83**, 4369-4392.
371. D. C. Essaka, J. Prendergast, R. B. Keithley, M. M. Palcic, O. Hindsgaul, R. L. Schnaar and N. J. Dovichi, *Anal. Chem.*, 2012, **84**, 2799-2804.
372. C. Cecala and J. V. Sweedler, *The Analyst*, 2012, **137**, 2922-2929.

373. V. Lecault, A. K. White, A. Singhal and C. L. Hansen, *Curr Opin Chem Biol*, 2012, **16**, 381-390.
374. R. L. Fernandes, M. Nierychlo, L. Lundin, A. E. Pedersen, P. E. P. Tellez, A. Dutta, M. Carlquist, A. Bolic, D. Schapper, A. C. Brunetti, S. Helmark, A. L. Heins, A. D. Jensen, I. Nopens, K. Rottwitt, N. Szita, J. D. van Elsas, P. H. Nielsen, J. Martinussen, S. J. Sorensen, A. E. Lantz and K. V. Gernaey, *Biotechnol. Adv.*, 2011, **29**, 575-599.
375. J. R. S. Newman, S. Ghaemmaghami, J. Ihmels, D. K. Breslow, M. Noble, J. L. DeRisi and J. S. Weissman, *Nature*, 2006, **441**, 840-846.
376. G. W. Osborne, in *Recent Advances in Cytometry, Part A: Instrumentation, Methods, Fifth Edition*, eds. Z. Darzynkiewicz, E. Holden, A. Orfao, W. Telford and D. Wlodkowic, Editon edn., 2011, vol. 102, pp. 533-556.
377. S. C. Bendall, G. P. Nolan, M. Roederer and P. K. Chattopadhyay, *Trends in Immunology*, 2012, **33**, 323-332.
378. R. N. Zare and S. Kim, in *Annual Review of Biomedical Engineering, Vol 12*, Annual Reviews, Palo Alto, Editon edn., 2010, vol. 12, pp. 187-201.
379. H. Yun, K. Kim and W. G. Lee, *Biofabrication*, 2013, **5**.
380. X. Mu, W. F. Zheng, J. S. Sun, W. Zhang and X. Y. Jiang, *Small*, 2013, **9**, 9-21.
381. J. B. Wang, H. C. Fan, B. Behr and S. R. Quake, *Cell*, 2012, **150**, 402-412.
382. J. Sun, M. D. Masterman-Smith, N. A. Graham, J. Jiao, J. Mottahedeh, D. R. Laks, M. Ohashi, J. DeJesus, K. Kamei, K. B. Lee, H. Wang, Z. T. F. Yu, Y. T. Lu, S. A. Hou, K. Y. Li, M. Liu, N. G. Zhang, S. T. Wang, B. Angenieux, E. Panosyan, E. R. Samuels, J. Park, D. Williams, V. Konkankit, D. Nathanson, R. M. van Dam, M. E. Phelps, H. Wu, L. M. Liau, P. S. Mischel, J. A. Lazareff, H. I. Kornblum, W. H. Yong, T. G. Graeber and H. R. Tseng, *Cancer Res.*, 2010, **70**, 6128-6138.
383. Y. Wang, Z. Z. Chen and Q. L. Li, *Microchimica Acta*, 2010, **168**, 177-195.
384. A.-K. Gustavsson, D. D. v. Niekerk, C. B. Adiels, F. B. d. Preez, M. Goksör and J. L. Snoep, *Febs J.*, 2012, **279**, 2837-2847.
385. M. Zagnoni and J. M. Cooper, in *Recent Advances in Cytometry, Part A: Instrumentation, Methods, Fifth Edition*, eds. Z. Darzynkiewicz, E. Holden, A. Orfao, W. Telford and D. Wlodkowic, Editon edn., 2011, vol. 102, pp. 25-48.
386. T. P. Lagus and J. F. Edd, *J. Phys. D-Appl. Phys.*, 2013, **46**.
387. A. J. Mach, O. B. Adeyiga and D. Di Carlo, *Lab Chip*, 2013, **13**, 1011-1026.
388. A. K. White, M. VanInsberghe, O. I. Petriv, M. Hamidi, D. Sikorski, M. A. Marra, J. Piret, S. Aparicio and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 13999-14004.
389. K. Yasuda, A. Hattori, H. Kim, H. Terazono, M. Hayashi, H. Takei, T. Kaneko and F. Nomura, *Microfluid. Nanofluid.*, 2013, **14**, 907-931.
390. H. Yin and D. Marshall, *Curr Opin Biotechnol*, 2012, **23**, 110-119.
391. D. Desmaele, M. Boukallel and S. Regnier, *J Biomech*, 2011, **44**, 1433-1446.
392. M. Ni, W. H. Tong, D. Choudhury, N. A. A. Rahim, C. Iliescu and H. Yu, *Int. J. Mol. Sci.*, 2009, **10**, 5411-5441.
393. R. Fior, S. Maggiolino, M. Lazzarino and O. Sbaizero, *Microsyst. Technol.*, 2011, **17**, 1581-1587.
394. J. Castillo, M. Dimaki and W. E. Svendsen, *Integr. Biol.*, 2009, **1**, 30-42.
395. L. A. Devriese, A. J. Bosma, M. M. van de Heuvel, W. Heemsbergen, E. E. Voest and J. H. Schellens, *Lung cancer*, 2012, **75**, 242-247.
396. T. Xu, C. T. Shu, E. Purdom, D. Dang, D. Ilsley, Y. Guo, J. Weber, S. P. Holmes and P. P. Lee, *Cancer Res*, 2004, **64**, 3661-3667.
397. W. J. Choi, D. I. Jeon, S. G. Ahn, J. H. Yoon, S. Kim and B. H. Lee, *Opt. Express*, 2010, **18**, 23285-23295.

398. B. Vanherberghen, P. E. Olofsson, E. Forslund, M. Sternberg-Simon, M. A. Khorshidi, S. Pacouret, K. Guldevall, M. Enqvist, K. J. Malmberg, R. Mehr and B. Onfelt, *Blood*, 2013, **121**, 1326-1334.
399. A. Stahlberg, D. Andersson, J. Aurelius, M. Faiz, M. Pekna, M. Kubista and M. Pekny, *Nucleic Acids Research*, 2011, **39**.
400. J. Ji, F. Yu, Q. Ji, Z. Li, K. Wang, J. Zhang, J. Lu, L. Chen, Q. E, Y. Zeng and Y. Ji, *Hepatology*, 2012, **56**, 332-349.
401. S. I. Kim and H. I. Jung, *J. Breast Canc.*, 2010, **13**, 125-131.
402. L. Yu, S. R. Ng, Y. Xu, H. Dong, Y. J. Wang and C. M. Li, *Lab Chip*, 2013, **13**, 3163-3182.
403. J. Chen, J. Li and Y. Sun, *Lab Chip*, 2012, **12**, 1753-1767.
404. H. Choi, K. B. Kim, C. S. Jeon, I. Hwang, S. Lee, H. K. Kim, H. C. Kim and T. D. Chung, *Lab Chip*, 2013, **13**, 970-977.
405. K. R. Willison and D. R. Klug, *Curr Opin Biotechnol*, 2013, **24**, 745-751.
406. P. Dalerba, T. Kalisky, D. Sahoo, P. S. Rajendran, M. E. Rothenberg, A. A. Leyrat, S. Sim, J. Okamoto, D. M. Johnston, D. L. Qian, M. Zabala, J. Bueno, N. F. Neff, J. B. Wang, A. A. Shelton, B. Visser, S. Hisamori, Y. Shimono, M. van de Wetering, H. Clevers, M. F. Clarke and S. R. Quake, *Nat. Biotechnol.*, 2011, **29**, 1120-U1111.
407. D. Ramskold, S. Luo, Y. C. Wang, R. Li, Q. Deng, O. R. Faridani, G. A. Daniels, I. Khrebtukova, J. F. Loring, L. C. Laurent, G. P. Schroth and R. Sandberg, *Nat Biotechnol*, 2012, **30**, 777-782.
408. S. C. Bendall and G. P. Nolan, *Nat. Biotechnol.*, 2012, **30**, 639-647.
409. S. Esumi, S. X. Wu, Y. Yanagawa, K. Obata, Y. Sugimoto and N. Tamamaki, *Neuroscience research*, 2008, **60**, 439-451.
410. A. Kawaguchi, T. Ikawa, T. Kasukawa, H. R. Ueda, K. Kurimoto, M. Saitou and F. Matsuzaki, *Development*, 2008, **135**, 3113-3124.
411. J. M. Trimarchi, M. B. Stadler and C. L. Cepko, *PloS one*, 2008, **3**, e1588.
412. R. Lu, N. F. Neff, S. R. Quake and I. L. Weissman, *Nat. Biotechnol.*, 2011, **29**, 928-U229.
413. O. I. Petriv, F. Kuchenbauer, A. D. Delaney, V. Lecault, A. White, D. Kent, L. Marmolejo, M. Heuser, T. Berg, M. Copley, J. Ruschmann, S. Sekulovic, C. Benz, E. Kuroda, V. Ho, F. Antignano, T. Halim, V. Giambra, G. Krystal, C. J. F. Takei, A. P. Weng, J. Piret, C. Eaves, M. A. Marra, R. K. Humphries and C. L. Hansen, *Proc. Natl. Acad. Sci. U. S. A.*, 2010, **107**, 15443-15448.
414. L. Z. Shi, J. M. Nascimento, C. Chandsawangbhuwana, E. L. Botvinick and M. W. Berns, *Biomed Microdevices*, 2008, **10**, 573-583.
415. M. Iskar, G. Zeller, X. M. Zhao, V. van Noort and P. Bork, *Curr Opin Biotechnol*, 2012, **23**, 609-616.
416. L. Flatz, R. Roychoudhuri, M. Honda, A. Filali-Mouhim, J. P. Goulet, N. Kettaf, M. Lin, M. Roederer, E. K. Haddad, R. P. Sekaly and G. J. Nabel, *Proc. Natl. Acad. Sci. U. S. A.*, 2011, **108**, 5724-5729.
417. M. T. Dieterlen, K. Eberhardt, A. Tarnok, H. B. Bittner and M. J. Barten, *Methods Cell Biol*, 2011, **103**, 267-284.
418. M. A. Khorshidi, B. Vanherberghen, J. M. Kowalewski, K. R. Garrod, S. Lindstrom, H. Andersson-Svahn, H. Brismar, M. D. Cahalan and B. Onfelt, *Integr. Biol.*, 2011, **3**, 770-778.
419. M. Walde, J. Moneypenny, R. Heintzmann, G. E. Jones and S. Cox, *PloS one*, under review.