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SPECTROSCOPIC IMAGING: A SPATIAL ODYSSEY.

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Abstract

Analytical methods were developed or refined to link the composition and structure of man-made and natural materials down to the nanoscale dimensions to their functional behaviour at the macroscopic scale. Most of the techniques now available are based the interaction of the sample with electromagnetic radiation or particles. In these techniques, the ability for observation and analysis expanded over the last 50 years from rather straightforward measurements in the narrow wavelength range in, or near to, the visible region, to large portions of the electromagnetic spectrum and complex higher-order interactions in multi-spectral and hyper-spectral imaging methods. With imaging analysis and other analytical methodologies that produce massive amounts of data, analytical chemistry is increasingly transformed into a discovery-driven, shotgun methodology, instead of a hypothesis-driven, targeted methodology. In such conditions, standard metrological concepts (uncertainty, validation, and/or traceability to fundamental standards…) lose their central guiding role.

Brief

The crossroads between imaging and spectroscopy gives rise to spatial analysis, an exciting new sub-discipline of analytical chemistry

Introduction

The crossroads between microscopic imaging as an observational tool and spectroscopy as a tool for chemical analysis occurred in the early 1960s and led to an incremental development process with the emergence and successive refinement of a variety of specialized analytical techniques that, over time, matured into a set of versatile and powerful tools for visualizing structural and compositional heterogeneity. These methods are now opening up new opportunities for the study of diverse heterogeneous materials. These increasingly find applications in nanotechnology and in the detailed study of natural objects. Over time, imaging analytical techniques evolved first to the microscopic size level, then to the mesoscopic (the 100-1,000 nm range) and, more recently, to the nanoscale level, down to a few nm **[1,2]**. The ultimate goal is chemical imaging with the highest possible spatial resolution with methods and instrumentation that is able to identify and accurately measure as little as a few thousand, even individual atoms and molecules. In imaging analysis, the measurements by themselves are not sufficient for their proper interpretation, they must be related to the physico-chemical system sampled in terms of concentration and/or spatiotemporal localization. The Odyssey in the title refers to the ancient Homer's work.

The lay-out of this classical epic is a travel account of Odysseus journey home, then of his adventures for re-establishing order in his homeland, after a 20 years long stay abroad. Following the structure of this work, we will first sketch the evolution of imaging analysis from its start in the early 1960s to the present, after which we will discuss the impact of this new sub-discipline on analytical chemistry.

Imaging analytical techniques

At present, there exists an impressive range of mature analytical methods for the analysis of heterogeneous objects, inclusions, surfaces, thin-film layers and interfaces. The presently available analytical methods provide essential information on atomic or molecular composition, with a sensitivity and specificity which is unrivaled by conventional techniques. Most available methods rely heavily on the use of intense focused electromagnetic radiation or particle beams.

The development of imaging analytical techniques came at the price of increasingly complex equipment and methods of analysis. The data resulting from systematic localized measurements, as pursued in imaging analysis, consists of large amounts of information covering an ensemble of elements of a two-dimensional (2-D) data set or a three-dimensional (3-D data) data array. Every measurement point may consist of diverse various data: numbers representing analytical results with their significance levels, but also patterns such as diffraction sets, morphological information, etc. In addition, due to its applications, imaging analytical chemistry is increasingly becoming a discovery-driven, shotgun methodology, instead of a hypothesis-driven, targeted one.

In what follows we will cover briefly the salient facts of the evolution of spectroscopic imaging analysis. We will first give an overview of imaging analysis, how it evolved, from the macro to the nano size level, where it is today. Then we will go in some detail on the profound implications this has on analytical chemistry as a field of research: what is the role of imaging analysis in science and technology? How to reach compliance between analytical results?

The Odyssey of spatially resolved spectroscopic imaging.

The historical development of imaging analysis, also called analytical imaging, chemical imaging, spatial analysis, elemental or molecular mapping, is schematically represented in Figure 1. The figure shows three distinct phases of development: first, until around 1960 the period of analytical chemistry as a macro scale methodology, separated from optical microscopy as an observational methodology with diffraction limited spatial resolution, then around 1960, the combination of both in instrumental imaging analytical tools (electron microscopy, beam techniques, probe techniques…) with an extended spectral range and the development of surface analysis, 2-D and 3-D analysis, (tomography, depth profiling). By the turn of the century, imaging analysis extended the analytical potential from the microscopic scale into the nanoscopic imaging analysis with various forms of hyperspectral analysis, super-resolution methods and combined instruments with full and automated data analysis and data management. Analysis at the nm level became possible, in some techniques reaching the single atom/molecule detection level. The history of imaging analysis is dominated by two paradigm shifts, first through the combination of imaging and analytical tools, then as a result of the breakthrough of nano-analytical potential and super-resolution imaging analysis **[3]**.

The evolution of imaging analysis

A number of factors can be recognized that contributed to the development of imaging analysis into the microscopic and sub-microscopic domains. Some issue from elementary scientific facts, such as the dual nature of light with the diffraction limit of radiation, or from elementary three dimensional Euclidean metrics, when the sample (pixel or voxel) size is confronted with the magnitude of Avogadro's number. High spatial resolution needs high analytical sensitivity: every order of magnitude in 3-D spatial discrimination requires a sensitivity enhancement by three orders of magnitude, pushing the absolute detection limits rapidly from the nanogram, to the picogram, the femtogram and the attogram levels as spatial resolution moves from the microscopic to the nanoscopic level.

At present, with some imaging tools we arrived at the extreme limits of analytical chemistry: the characterization of attomole to zeptomole quantities of atoms or molecules. At a sampling level of $1nm^3$ ($10^{-18}cm^3$), there are simply not enough sample atoms or molecules left for reaching any realistic relative sensitivity levels. This means also that the recovery of analyte information and detection efficiency need to be optimized to maximum performance levels. In dynamic secondary ion microscopy (SIMS) with the CAMECA instruments, for instance, sputtering will analyse volumes of 50 x 50 X 10 nm containing typically 1.2 million atoms. With an ionisation of 0.005 and a transmission and detection efficiency of unity, one can detect *ca* 6,000 atomic ions, or a detection limit of roughly 0.1 % **[4]**. SIMS being a destructive technique – the material is sputtered away and lost - there is no possibility to resolve this problem by increasing the measurement time.

Abbe-Rayleigh diffraction limit.

In 1873, Ernst Abbe postulated that diffraction fundamentally limited the resolution that the microscope could achieve to *ca* half the wavelength of the light used. In a more rigorous treatment than that made by Abbe, Lord Rayleigh defined the minimal resolvable distance between two equal brightness point sources imaged by a diffraction-limited imaging system **[5]** as:

$d = \lambda/(2n \sin \theta) > 200$ nm   (1)

with n, the refractive index of the lens medium and θ, the half angle over which the objective gathers light from the specimen ($n \sin \theta$ is the numerical aperture, NA, of the lens) and λ the wavelength in nm

of the light source used (visible 400 to 740 nm). Two objects that are separated by a distance less than d, for near-UV visible light *ca* 200 nm, cannot be distinguished from each other and will appear as a single object when viewed with any optical system.

Near-field optical approaches overran the obstacle of the Abbe-Rayleigh diffraction limit for visible light and lowered the detection area or volume beyond the limit imposed by the diffraction for visible light, with approaches such as Scanning Near-field Optical Microscopy (SNOM) or Tip-Enhanced Raman Spectroscopy (TERS). Non-linear (e.g. two-photon) and super-resolution optical spectroscopic techniques were also developed and led to substantial improvements in image resolution. For example, Stimulated Emission Depletion (STED) microscopy broke the diffraction barrier by inhibiting the fluorescence emission of a fluorescent marker [6]. Methods such as Stochastic Optical Reconstruction Microscopy (STORM) [7], Photo-Activated Localization Microscopy (PALM) [8] and a large series of other fluorescence related optical techniques (there are now at least a dozen more) opened up biological applications at the nano spatial level for the analysis of single cells and sub-cellular components. A recent review gives a thorough overview on the rapid growth of optical super-resolution imaging [9].

The race against the diffraction limit also explains the use of elementary particle beams classified as "beam" analytical techniques. For particles, the equivalence between particles and radiation (the de Broglie wavelength) can be expressed as:

$$\lambda = 1239.8 \text{ (eV.nm) } /(2.E_{kin}.m_o c^2)^{0.5} \tag{2}$$

where E_{kin} is the kinetic energy of the particle and $m_o c^2$, its rest mass energy in eV.

For a small particle of low energy, say an electron with kinetic energy 1 eV the de Broglie wavelength thus corresponds to 1.24 nm, about a factor 1,000 smaller than for a 1eV (1240 nm, infrared) photon.

Beam techniques are applied over an enormous range of particle mass and energy: from low energy electrons to energetic massive projectiles, such as e.g. Au_{400}^{4+} ions. The impact of such particles gives rise to the emission of a series of secondary particles ranging from electrons to neutral and ionized atoms, molecules and molecular fragments. The bombarding particles provide, dependent on experimental circumstances, spatially localized and in-depth information from the measurement.

Electron microscopy derives its utility in high resolution imaging from its de Broglie wavelength - and its associated diffraction limit - but is limited through aberration effects to somewhere in the 50 picometer range, small enough to measure the spatial position of individual atoms. The same applies for the use of protons in Ion Beam Analysis (IBA) in Particle Induced X-ray Emission (PIXE) and in various elemental and polyatomic or cluster ions as bombarding projectiles in SIMS. Laser irradiation of shorter than visible wavelength is also used for laser ablation (LA) of material in Inductively Coupled Plasma Mass Spectrometry (ICPMS) and for ionization of elemental ions and molecular fragments in Laser Induced Breakdown Spectrometry (LIBS). All these methodologies are limited in spatial resolution by the beam size used, instrumental shortcomings or effects related to the sample interaction, rather than by the intrinsic limitation due to diffraction.

Among the many alternative methods that are available now, a number of prominent imaging analytical techniques stand out for their current prominent role in analytical chemistry and in various scientific fields. Transmission/Scanning Electron Microscopy (TEM, SEM) and Electron Probe Micro-Analysis (EPMA) are able to provide elemental information through X-ray emission spectrometry and speciation

through techniques such as electron energy loss spectrometry (EELS). Dynamic and Static SIMS (D-SIMS and S-SIMS) **[10]** are now able to measure a wide range of elements and organic species at close to the level of the individual atom or molecule, a few tens of a nanometer. Laser Microprobe Mass Spectrometry (LMMS) is used in elemental and organic imaging applications. LA-ICPMS **[11]** has gained widespread popularity for elemental imaging due to its wide multi-element capacity, large dynamic range, ultra-trace sensitivity, potential for isotope analysis and spatial resolution down to ca. 15-50 μm^2 .

Soft ionization plasma techniques were transformed into important imaging tools at the spatial resolution level of a few tens of the micrometer for organic, bio-analytical and various "-omics" related methodologies. Imaging mass spectrometric methodologies such as Electro-Spray Ionization (ESI), Matrix-Assisted Laser Desorption Ionization (MALDI), Collision-Induced Dissociation (CID) are but a few examples of a wide range of related techniques **[12]**. They all require completely new paradigms in which it becomes possible to manipulate and separate ions in complex ways, in order to detect them, sometimes in parallel MS systems. IBA, using nano size particle beams combined with techniques such as micro-PIXE and Rutherford Backscattering Spectroscopy (RBS) and related techniques such as Elastic Recoil Detection (ERD or ERDA) provides mapping possibilities at the spatial level of a few tens of a nanometer. Microscopic X-ray Fluorescence analysis (micro-XRF) and related inner-shell ionization methods based on X-ray absorption are exploited in synchrotron radiation facilities at similar levels of spatial resolution and provide also 3-D (tomographic) analytical potential. Electron Photoelectron Spectroscopy (XPS, ESCA), scanning Auger Electron Spectroscopy and Scanning Auger Microscopy (AES, SAM) are other techniques to analyse the heterogeneity of samples.

Micro- and nano-imaging

The aim of imaging analysis is to derive compositional information, but it requires also morphological or structural information for the interpretation of the data. Major advances were made over the last decade for structural imaging. The most frequently used technique for diffraction imaging is Selected Area Electron Diffraction (SAED or SAD) which relies on the registration of the diffraction pattern of a sample under broad parallel electron illumination with an aperture in the image plane. The fully automatic creation of orientation maps by Electron Back Scattered Diffraction (EBSD) on the basis of channeling patterns in the SEM is now available commercially as a well-established method.

The conventional approach for X-ray imaging relies on x-ray absorption as the sole source of contrast and draws exclusively on geometrical optics to describe and interpret image formation. This approach ignores another, potentially more sensitive source of contrast - phase information of the wavefront. Phase retrieval approaches provide measurements of the local phase shift, which is proportional to the density variations in the sample. For low atomic number samples (e.g. biological samples) these are considerably more sensitive for detecting small compositional differences than the absorption. Research is going on since at least a decade now, in which ways are explored for exploiting phase information as a source of image contrast, using approaches that fall into three broad categories: interferometry, diffractometry, and in-line holography. At a fundamental physics level, these three modes are associated with directly measuring of the phase change introduced in a coherent incident X-ray beam on passing through the sample.

Imaging analysis and nanotechnology

Nanotechnology brought new imaging tools such as Scanning Tunneling Microscopy (STM), Atomic Force Microscopy (AFM) and various derived techniques. They are used increasingly as observational tools together with spectroscopic imaging analysis. The use of nanomaterials as analytical tools - applying them for analysis - is also at present of major concern. Metallic nanoparticles can be used to increase the sensitivity of fluorescent detection because they generate a phenomenon known as metal-enhanced fluorescence, thus increasing fluorescence lifetime and quantum yields [14]. They also result in localized surface plasmon resonance (LSPR). Quantum dots have the potential to solve many of the problems associated with organic fluorophores in near infrared spectroscopy. Their inorganic core and shell enables tuning of fluorescence with a narrow bandwidth enabling multiplexed detection of molecular targets **[13]**.

Raman spectroscopy is a particularly good example of the profound changes introduced by nanotechnology. As an analytical technique Raman spectrometry is normally not sufficiently sensitive for many applications, and certainly not for imaging applications. Through surface enhanced Raman spectroscopy (SERS) it gained tremendously in sensitivity but this electromagnetic enhancement could for decades not be exploited because of the erratic behaviour of the phenomenon. With well characterized nano-size particles or a nano-size STM tip this situation has recently been changed by simultaneously locally enhancing the excitation field with a "plasmonic nanoantenna" while using a spatial confinement with a nano-aperture. In such conditions fluorescent and Raman enhancement becomes important enough to allow single molecule detection **[14]**.

Individual impacts of beams of clustered particles that are focused to < 3 nm diameter, can be used to detect analytes within nanometric volumes. Co-ejected ions from a single impact can be detected by time-of-flight mass spectrometry and reveal the presence of co-located molecules within a short distance of a few nm **[15]**. Co-localization is an important concept and relates to the spatially close connection between specific structural characteristics and analytical signals (e.g. trace elements located near a dislocation in a semiconductor) or the close position of molecules in an heterogeneous object, e.g. two biomolecules or a biomolecule and organelles or vesicles in cells, separated by a distance of ≤ 2 nm, a distance where they may give rise to chemical interaction.

Alternative approaches for molecular co-localization employ fluorescence detection. Fluorescence microscopy/spectroscopy based on Fluorescence (or Förster, for the initiator of the methodology**)** Resonance Energy-Transfer techniques (FRET) can be used to measure separation distances between 1 and 10 nm **[16]**. Although FRET is clearly analytical chemistry and analytical imaging, its development and applications are completely situated within the realm of biophysics.

At least five, perhaps there are already more, analytical imaging techniques achieve at present the ultimate detection potential of one single atom or a discrete molecule: high resolution TEM, 3-D atom probe tomography, TERS, fluorescence microscopy and time-of-flight ERDA analysis. With chemical analysis at this ultimate spatial resolution of a single atom or a single molecule, it becomes possible to count the number of atoms or molecules in a given volume. In such circumstances, the analytical chemistry aspects are reduced to recognizing false positives and false negatives and proper arithmetic's. Simple statistics determine the uncertainty level of the measurement **[17]**. Speciation information depends on the detection of neighbouring elements. In reality, spatially confined analysis is more complicated, however, as we will discover in the next section.

Imaging analysis as a quantitative analytical methodology.

As in any other quantitative analytical technique, imaging analysis is typically achieved by relating the intensity of a particular spectral feature to the concentration of one or more analytes in the sample. Calibration is important to ensure precision in the series of measurements which together constitute the image. Extracting quantitative information is difficult due to matrix effects and other instrumental factors affecting the measurements; these are particularly important for samples with a high degree of complexity and heterogeneity. Therefore, for many of the chemical imaging techniques it is difficult to obtain reliable quantitative information. Some methods are becoming highly sensitive and spatially selective but remain semi-quantitative or qualitative tools. It is clear that more research is required for fully understanding all the spectroscopic information, especially in complex environments such as biological systems. Moreover, there is need for certified materials that enable calibration at high spatial and depth resolution. Until now, few CRMs or RMs suitable for laterally defined analysis have been produced. Various other instrumental artifacts (edge effects, effects resulting from the combination of images in an overall larger scale on, tiling…) in the techniques discussed may produce reproducibly distortions in the imaging data. It is important to generate tools to correct for such artifacts after the data acquisition data collection. Multi-order correction algorithms to remove image distortions, e.g. in mass spectrometric imaging datasets (in time-of-flight SIMS) have been developed [18].

Overall, there remains a lot of potential for development of a comprehensive system of quality assurance; method validation is difficult. Performance characteristics related to precision and accuracy differ between the different methodologies that are available today, with elemental spatial analysis being most advanced for obtaining quantitative information.

X-ray fluorescence (or emission) is the most accurate chemical imaging tools. In principle, the processes governing the creation of fluorescence radiation and their absorption in sample and detector are well understood, and fundamental parameter based methods are able to derive to yield reliable quantitative data. Reference-free quantitative analysis with XRF analysis requires *a priori* knowledge on relevant experimental and fundamental parameters associated with the atomic inner-shell photo-ionization processes and subsequent radiative and non-radiative transitions. These fundamental parameters include photo-ionization cross-sections, X-ray, Auger and Coster-Kronig transition rates, fluorescence yields and vacancy transfer probabilities. Extensive research was made in the past for the experimental determination or calculation of the parameters involved and these are available, but distributed throughout the literature [19]. Several metrology devoted beamlines at synchrotron radiation facilities allow performance studies with this powerful sources, aimed at improved accuracy analysis. As such, there is no reason why X-ray imaging analysis methods could not join the chemical metrology Olympus with the primary (traceable) methods of analysis. The X-ray techniques could become important as a potential primary method of analysis and a tool for the fabrication of CRMs and RM for other imaging analysis techniques.

The development of quantitative molecular imaging techniques has proven to be a lot more difficult. Techniques such as MALDI analysis require the sample surface be coated with matrix, typically a small organic compound that facilitates the analyte's extraction and ultimately, the "soft" ionization of desorbed molecules. As such the matrix enhancement is both a help to boost sensitivity but simultaneously a limiting factor for reliable quantitative analysis. Ellis et al. recently published a critical evaluation of the current state-of-the-art in quantitative imaging mass spectrometry. They conclude that these methods remains a significant analytical challenge but highlight the significant progress that is being made in the field [20]. Advances are largely related to the development and optimization of

internal standard based signal calibration protocols that allow the correlation of ion intensities to an absolute surface concentration. A similar situation occurs for SERS with the "plasmonic nanoantenna" referred to earlier. The plasmon enhancement effect provides a tremendous gain in sensitivity but it remains difficult to quantify the SERS signal because of irreproducible amplification. Nevertheless, quantitative Raman analysis was recognized as a metrological challenge and a number of international Measurement Institutes joined forces in a project "Metrology for Raman Spectroscopy" to improve Raman spectroscopy quantification using SNOM and TERS **[21].**

 Imaging analysis: a complex data gathering tool

Imaging analysis methodologies are based on the systematic extension from one-dimensional (point, 0-D) observations to a line (1-D), and then to a surface to produce two-dimensional (2-D) images to obtain finally 3-D information through either combining successive 2-D images or more directly by tomography. The goal of this process is to obtain information on the spatial modulation of mass density, structure and composition of the object under study, or even the temporal variation of such information, including time as a fourth dimension (4-D). This fourth dimension transforms imaging analysis in a cinematographic experiment, the dynamic observation of changes in composition, structure or morphology over time.

Each element of a three-dimensional array can be considered as an item of numerical information representative of the concentration of one or more analytes in the heterogeneous object. It can also be more complicated and may consist of an elaborate set of data, e.g., a complex high-resolution mass spectrum, including sets of collision-induced fragments, or a hyper-spectral set of data resulting from the combined use of various observational tools. In this case, the result is a data cube for every measurement point, with a range of structured or sometimes unstructured information. Each such element becomes a data collection of increasing complexity and size to deal with. The information in each data cube may consist of various types of data, numbers with their significance level, images such as diffraction pattern sets, images representing morphological information, textual data, etc.

Finding the best way to share and interpret the results of such data sets can be difficult, but new methods are constantly emerging. Indeed, such complex data aggregates are not unique to imaging analysis: for instance, one can mention the ever-increasing volume of information obtained in chromatography-related (hyphenated) platforms of analysis for speciation analysis, molecular analysis, or the many techniques used in proteomics and metabolomics...

Integrated approaches in imaging analysis

In order to understand the link between composition and structure at the nano/microscopic scale and functional behaviour at the macroscopic scale, it became necessary to vastly increase the information content of the analytical process. This is brought about by the simultaneous measurement of various beam-sample interaction processes or by using increasingly large parts of the electromagnetic spectrum. There also are numerous approaches in which previously distinct analytical methods have been combined, either in a single instrumental setup, or as a combination of several instruments. Examples include but are not limited to:

- SEM in combination with integrated Electron X-ray Emission (EDX) and EBSD **[22]**;

- the combination of light, fluorescence with fluorescent dyes and electron microscopy in Correlative Light-Electron Microscopy (CLEM) **[23]**;
- the combination of various X-ray matter interaction modes in Micro- X-ray fluorescence analysis **[24]**, X-ray Diffraction (XRD), X-ray Emission Optical luminescence (XEOL) **[25]**, together with absorption and edge scanning techniques for speciation such as XANES and structure such as Extended X-ray Absorption Fine Structure Analysis (EXAFS) in synchrotron X-ray analysis;
- the combination of Atom Probe Tomography (ATP), Field Emission Microscopy (FIM) and TEM **[26]**;
- various organic mass spectrometric methods in combination with ICPMS **[27]**;
- the combination of STM and ATM with various spectrometric or mass spectrometric techniques **[28]**;
- scanning X-ray analysis (XRF, XRD…), X-ray radiography, infrared reflectography, laser interference techniques, terahertz imaging, NMR, etc… for macro-analysis of paintings **[29]**.

Of particular importance in all these integrated approaches is the combination of imaging observational techniques, with imaging spectrometric (analytical) methods. The following example illustrates the issue further.

A spectacular example of nanoimaging and nanoanalysis

The European Synchrotron radiation facility (ESRF) is now in a major upgrade phase after 20 years of operation (first phase 2009 - 2015).The upgrade includes improvements to the X-ray source (stability, and source characteristics matching the instrument's diffraction limit) and eight new beamlines with nano-focussing optics, pixel detectors, on-line data analysis, high-rate data collection.

The acme of X-ray related instrumentation for imaging analysis is the new beamline UPBL4 ID 16, also called "NINA" that becomes available in 2014. NINA has two branches, one for nanoimaging (NI), the other for nanoanalysis (NA). Its mission consists in the full characterization at the nano-scale of heterogeneous samples with complementary methods for the study of both biological and materials science samples. Main fields of application for NI are biology and the life sciences, nanotechnology and nanomedicine; for NA they are biology, environmental sciences, geoscience and materials sciences. The multi-modal approach (allowing spectroscopy-diffraction-imaging) provides complementary information through XRF, X-ray absorption near edge spectroscopy (XANES and EXAFS), XRD and XEOL in the (incoherent, highly monochromatic and fully tunable) NA branch, and magnified holotomography, ptychographic coherent diffraction imaging (PCDI) and computed tomography in the (coherent, pink beam focus) NI branch. Elemental analysis and speciation should be possible with lower zeptogram detection limits in a 50 nm pixel **[30]**. Pushing the experimental conditions to the limit, the detection of individual atoms might become possible with the instrument.

The characteristics of the NINA instrumentation are summarized in Table I. There is an intention to include a TEM/SEM in the NI branch with vacuum transfer to the X-ray measurement position **[31]**.

Table I. The NINA beamline for imaging and analysis at the ESRF.

Characteristics	X-ray ultra-microscopy, nano-imaging (NI)	Nano-spectroscopy (NA)
Length	185 meter	165 meter

Spatial resolution	10-100 nm	50 nm – 1 mm
Focal spot size	16 x16 nm (projected), 29 x 29 nm (actual)	71 x 101 nm
Flux @ 18 keV	2.5 10*12 ph/s	1.8 10*12 ph/s
$\Delta E/E$ (%)	1	10^{-2}
Energy range	Discrete, 11 – 17 – 33 keV	Scanning, 5 → 70 keV
Main goals	XRF, coherent XRI-2D/3D, Cryo-environment; cryo-environment; integrated TEM/SEM as additional tools for imaging	XAS, XRD, XRF, XRI-2D/3D, combined *in-situ* experiments

Imaging analysis, in search for truth

Imaging data can be interpreted in different ways. A particular image might suggest directly a truth, but this is not always a reliable approach. One common way for representing and interpreting the data of an image is to attempt distinguishing those data with an inherent order from those without. Therefore, in imaging analysis data interpretation is focused on the differences between individual results and, in general, on patterns of data variability in the multivariate data set. The recognition of co-localization and patterns of elemental and molecular information with structural and morphological data are the issues of direct concern. The basis is the assessment of the difference between two or more observed measurements. The issue of concern in chemical imaging is the observation of the least significant difference between two population distributions while in conventional analytical chemistry it is the uncertainty of the analytical result compared to the true value.

Traditionally, chemical analysis is based on the formulation of a hypothesis about the composition of the object of study with regard to one or more constitutional components (the analytes, the measurands). The analysis is a targeted and a straightforward measurement process and, as such, is related to metrology, but should not be identified with chemical metrology. It should be designed and executed to provide accurate and purpose-specific compositional information in a direct sequence from the object to be analyzed to the analysis itself and the interpretation of the results (figure 2a). Validation of the results is based on the principles of chemical metrology, i.e., uncertainty, validation, and/or traceability, according to the definition of analytical chemistry as agreed by the International Union of Pure and Applied Chemistry (IUPAC) in 2002[1].

By contrast, in chemical imaging, the analysis evolves into considerably more complex problem-solving strategies. Numerous analyses need to be performed and evaluated in a systematic manner. The analysis involves observation of the object of analysis and possibly functional characteristics on one side

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[1] The IUPAC definition is as follows: Analytical chemistry is a scientific discipline that develops and applies methods, instruments, and strategies to obtain information on the composition and nature of matter in space and time, as well as on the value of these measurements, i.e., their uncertainty, validation, and/or traceability to fundamental standards

and compositional, sometimes structural analysis on the other side (figure 2b). The result is an often overwhelmingly large and complex collection of data that must be interpreted.

New paradigms are required to analyze the complex data sets resulting from the efforts to characterize, and ultimately understand, the natural world at the lowest structural level or to master man-made nanostructured materials.

Table II gives a schematic representation of the major points of difference between conventional chemical analysis and imaging analysis.

In an increasing number of present-day applications of analytical chemistry, imaging analysis and other forms of analysis, e.g. those based on hyphenated techniques, have become discovery-driven (shotgun approaches) rather than hypothesis-driven (targeted approaches). Such approaches begin with the collection of a large number of data on morphology, function, structure and composition. The evaluation of this complex set of data may or may not lead to the formulation of a hypothesis - depending on whether the experiments manage to provide an answer to the problem. The resulting hypothesis may then require a traditional targeted analysis of the sample (with chemical metrology as a guiding principle) **[32].** On the other hand, it is also possible that the resulting variability of the data set is sufficient for the purpose. In these conditions, standard metrological concepts lose their predominant position, they remain valid only when relevant for the issues at stake.

Table II. Imaging analysis compared to traditional analytical chemistry.

Chemical analysis, targeted analysis	Hypothesis-driven, deductive	Subject to rules of chemical metrology	Analytical result with confidence interval	Standard metrological concepts apply
Imaging analysis, non-targeted set of experiments	Data driven; Explorative; Hypothesis results from experiments, inductive	Complex data set; evaluation with statistical tools, chemometrics	Variability in composition, structure, morphology.	Metrological concepts lose central meaning, only of subsidiary relevance. Falsifiability becomes validation tool

The data deluge

The European Synchrotron Radiation Facility (ESRF) estimates that it generated approximately 1 petabyte ($1PB = 10^{15}$ bytes) of raw, mostly analytical data in 2011 and this data output is growing exponentially **[33].** Massive amounts of data are created not only by large science tools such as the synchrotron sources, but also by laboratory instruments, including analytical instrumentation. These data need to be collected, processed, analysed and visualised. In order to deal with the enormous flood of increasingly complex descriptive data produced by the new platforms for imaging analysis, there is a need for a more accurate planning of the measurements, and for the efficient extraction of relevant information. Information needs to be sifted, organized, compiled, and connected in such a way as to enable the formulation of predictions based on general principles. Such a development is greatly

facilitated by the staggering advances in computation power and data handling, but requires also a solid scientific basis.

In imaging analysis, data interpretation is focused on the differences between individual results and, in general, on patterns of data variability in the multivariate data set. The recognition of co-localization and patterns of elemental and molecular information with structural and morphological data are the issues of most concern. Chemometric tools for data analysis are available, with principal component analysis (PCA) and analysis of variance (ANOVA) as presently the most prominent methods. These methodologies must now be adapted to the needs of mega-variate data sets. Their main goal is to decrease the complexity of the images by reducing the number of variables. The simpler an image is, the more easily interpretable it will become. The largest variation in a dataset is likely to be the most relevant. Removing the irrelevant variation is the primary goal [34].

The development of new tools in chemometrics that are able to deal with the complexity of the datasets is an important task for the future. We need to be on guard, however, our eyes and our minds evolved over millions of years to recognize certain patterns as important, but these are not necessarily the right ones in context with the interpretation of the data.

The luxury of confidence

From the forgoing discussion it becomes clear that the reality of analytical chemistry in imaging analysis, and in other massive data gathering kinds of use (e.g. 1-D or 2-D chromatography...) is incommensurate with the IUPAC definition as it was defined in 2002. It is impossible, nor necessary, to keep metrological orthodoxy for all individual analytical results within the complex undertakings that are now employed in imaging analysis.

Either analytical imaging would not be an integral part of analytical chemistry or, else, more logically, the IUPAC definition of 2002, that defines our scientific discipline today, does not do justice to analytical chemistry as it develops in the 21st century. It defines the discipline way too narrowly, excluding not only imaging analysis from the realm of analytical chemistry, but also many other strategies to explore composition and structure in science and technology with the numerous methodologies that are available today.

Analytical chemistry needs to get a rational redefinition of its mission statement. Perhaps, this definition could just simply be: "analytical chemistry is the discipline that is concerned with the chemical composition of materials. Analytical chemistry also is concerned with developing the tools used to examine chemical compositions", a straightforward definition devised by Anne Marie Helmenstine [35]. As such, analytical chemistry escapes from being identified with chemical metrology, as a fiefdom of metrology. Its validation base then becomes the pursuit of truth, instead of the exactness of individual measurements as it is in the IUPAC definition, and this by whatever means that is necessary, including metrological concepts. Even despite this argument, I think that Helmenstine's direct and simple definition is preferable to the more elaborate one defined by the IUPAC.

With such a leading principle, epistemological concepts become central as validating tools. The basic question becomes: can experiments be repeated and verified? This resounds on the "falsifiability" concept of Karl Popper and his philosophy of scientific knowledge [36].

The philosophy of "consensus science" makes it possible to achieve compliance or agreement between analytical results - an idea whose significance goes beyond the analytical community itself **[37]**. While consensus science should be discussed as a future framework for scientific methodology in general, it becomes particularly important in analytical chemistry for its capacity to describe the heterogeneous structures of imaging analysis and other applications that generate large data sets.

Consensus building as a validation concept requires the full report of all experimental details and the data. Finding the best way to share all the results of a research project can be difficult and new ways need to be developed for reporting them in the literature, including negative and inconclusive results **[38]**.

Conclusion

Modern imaging technology has brought about a revolution in analytical imaging methodologies. Imaging analysis evolved into a new sub-branch of analytical chemistry, which could be named "*spatiation analysis*", by analogy with the now common use of the term "speciation analysis". Some of the techniques now available reach the nanoscopic domain and fundamental limits of atom or molecule detection.

This evolution comes at the price of increasingly complex and sophisticated equipment. Most of these instruments used in imaging analysis are automated, easy to use and include integrated data analysis instrumentation and software for the interpretation of the experimental data. When used in a non-targeted analytical approach, these analytical imaging methods create complex analytical data sets which require efficient tools for the extraction of relevant information. While in common applications analytical chemistry relies on the production of quantitative and accurate data, in imaging applications there is a need for increasingly complex data evaluation tools based on reliable statistical and chemometric tools.

Acknowledgments

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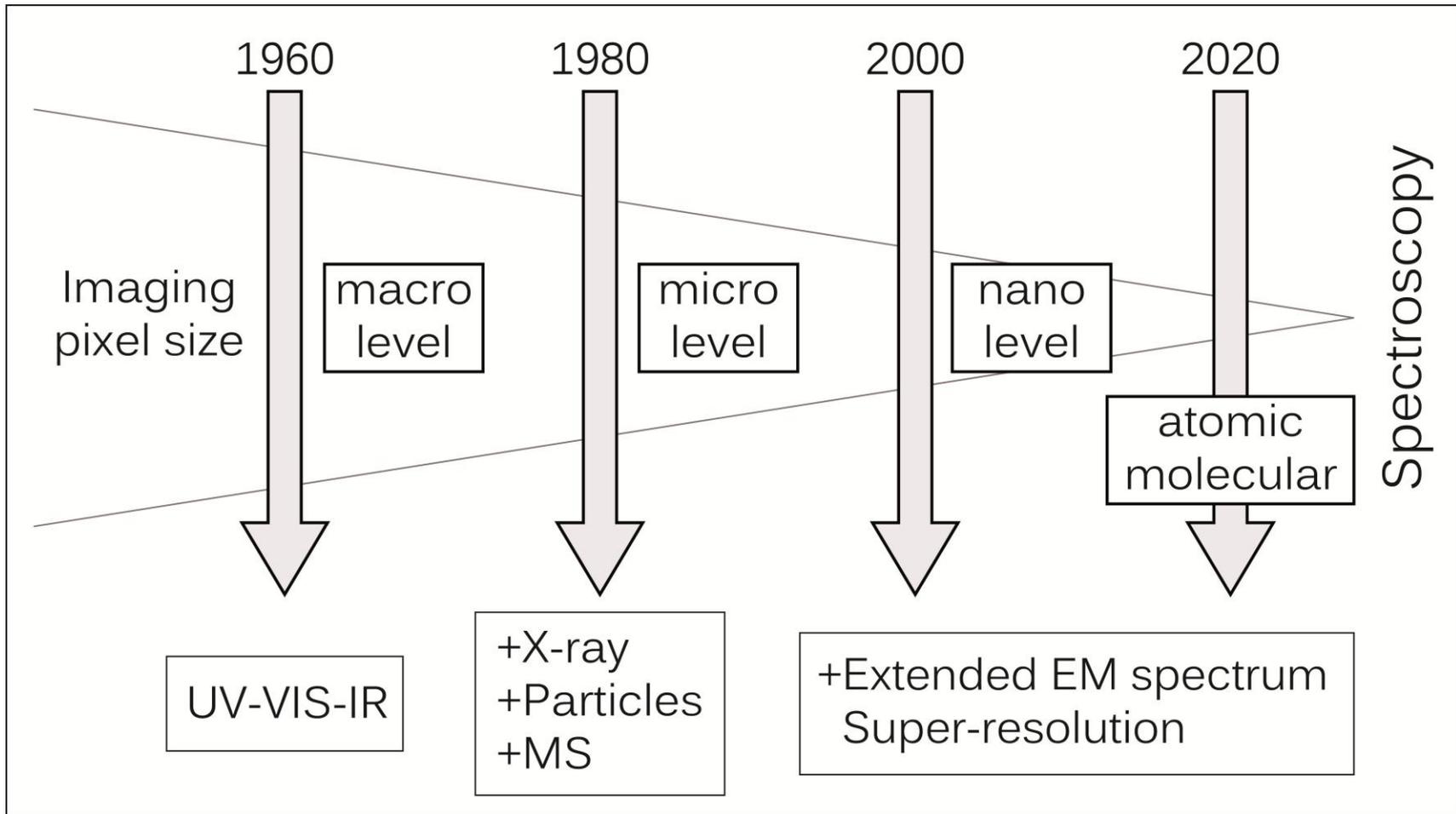[38] L. Mueck, Nature Nanotechnology, 2013, **8,** 693-695.

Figure captions

Figure 1. The Odyssey of spatially resolved spectroscopic imaging

Figure 2. The conventional targeted approach for chemical analysis (a), and the shotgun, non-targeted approach used in imaging analysis and other massive analytical methodologies (b).

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