Lab on a Chip

Enabling high-throughput single-animal gene-expression studies with molecular and micro-scale technologies

Journal:	*Lab on a Chip*
Manuscript ID	LC-PER-09-2020-000881.R1
Article Type:	Perspective
Date Submitted by the Author:	28-Oct-2020
Complete List of Authors:	Wan, Jason; Georgia Institute of Technology, Wallace H. Coulter Department of Biomedical Engineering; Emory University, Wallace H. Coulter Department of Biomedical Engineering Lu, Hang; Georgia Institute of Technology, Chemical and Biomolecular Engineering

Lab on a Chip

PERSPECTIVE

Enabling high-throughput single-animal gene-expression studies with molecular and micro-scale technologies

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Jason Wan[a] and Hang Lu [a,b,c*]

Gene expression and regulation play diverse and important roles across all living systems. By quantifying the expression, whether in a sample of single cells, a specific tissue, or in a whole animal, one can gain insights into the underlying biology. Many biological questions now require single-animal and tissue-specific resolution, such as why individuals, even within an isogenic population, have variations in development and aging across different tissues and organs. The popular techniques that quantify the transcriptome (*e.g.* RNA-sequencing) process populations of animals and cells together and thus, have limitations in both individual and spatial resolution. There are single-animal assays available (*e.g.* fluorescent reporters); however, they suffer other technical bottlenecks, such as a lack of robust sample-handling methods. Microfluidic technologies have demonstrated various improvements throughout the years, and it is likely they can enhance the impact of these single-animal gene-expression assays. In this perspective, we aim to highlight how the engineering/method-development field have unique opportunities to create new tools that can enable us to robustly answer the next set of important questions in biology that require high-density, high-quality gene expression data.

Introduction

Gene expression and regulation play diverse, critical roles ranging from defining phenotypic traits and heredity to the cause of stochastic emergent behaviors. It is only recently that technologies have advanced to test such hypotheses by directly measuring gene expression (*i.e.* mRNA content). We now know how crucial gene expression quantification is to biological investigations across multiple fields. From the types of surface proteins expressed during stem cell differentiation[1] to the changes of expression across the complex aging process of multi-cellular organisms[2], gene expression is a primary mechanism that can define the underlying biological processes occurring in the system. Gene expressions are the subject of intense research and are now critical to measure in many domains of biology.

As commonly performed assays to quantify gene expression have progressed from simple PCRs[3] to single-cell RNA-sequencing (scRNA-seq)[4], the types of biological questions we can ask have increased in complexity[5]. The tools and resources available to quantify transcriptome-level gene expression have become more accessible[6-8] and are pushing biologists to study gene expression with high spatial resolution on an individual level. Studies based on performing scRNA-seq on yeast[9-11], for example, have highlighted the importance of individual-level resolution and found possible implications of gene expression variability in the population (*i.e.* cell-to-cell or organism-to-organism variability). For instance, one study measured the expression variability at different ages[11] and showed an initial decrease in variability, which was followed by a period of increased variability and noise towards the end of the lifespan. This implied that gene expressions were maintained until a certain stage of life, which was then followed by an ultimate decline in regulation. The individual-to-individual variability revealed these overall shifts in genomic stability with age. While these examples provide insights, there are still questions as to whether this pattern is extended to multicellular organisms, and how we can prevent this loss of gene expression stability and possibly improve our healthspan (*i.e.* period of healthy living).

Along with individuality, it has become increasingly important to capture gene expression with spatial resolution. For example, studies comparing the transcriptomes of young and old mice[12] and rats[2] revealed changes in gene expression and highlight genetic pathways and networks that may be tied to certain perturbations. It is now known how gene expression is influenced by factors such as different diet[2], age[2, 13], or environmental stress[14]. Further questions ensuing might be: which tissues are more heavily influenced with age? Do individuals with better health and maintenance of those tissues or organs live longer? One might also want to ask, what types of cross-tissue interactions are present (*e.g.* how do gene networks interact between the gut and brain[15, 16])? While it is becoming more critical

[a] *Wallace H. Coulter Department of Biomedical Engineering, Georgia Institute of Technology and Emory University, Atlanta, Georgia 30332, USA*
[b] *School of Chemical & Biomolecular Engineering, Georgia Institute of Technology, Atlanta, Georgia 30332, USA*
[c] *Petit Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia 30332, USA*
**Author to whom correspondence should be addressed. Email: hang.lu@gatech.edu*

to address these disease/health-relevant questions, our current tools and methodologies may be the bottlenecks.

Methods to measure gene expression in multi-cellular organisms or tissues, such as traditional RNA-seq, have been very powerful in addressing developmental biology questions. Yet, there are limitations to these techniques – they often fail to capture mRNA content on a single-animal level or with tissue specificity. Although it may seem that we have the ability to quantify the transcriptome of an animal with single-cell resolution (in *Caenorhabditis elegans*[17]) or within specific tissues (such as in rats[2, 13] and mice[12, 18, 19]), there are inherent limitations due to the sample processing. Specifically, these pooled-sample techniques, such as RNA-seq, cannot measure two key components: gene expression on an individual level and the spatial expression information. With more and more investigations highlighting the importance of this type of information[14, 20-25], there is a growing need for single-animal, tissue-specific gene expression-based studies and tools.

Although there are single-animal assays such as fluorescent transcriptional reporters and single molecule *in situ* hybridization (smFISH), these techniques are very low throughput (both in terms of the number of genes studied and sample size) and thus cannot be easily used to study large populations or distinguish subtle phenotypes in noisy biological settings. To address the limitations of single-animal techniques, engineers have created new tools to improve sample handling, reagent delivery, and/or culturing conditions for animals. While diverse fields have made meaningful contributions and impacts, it is important to highlight that many of these achievements are based on microfluidic technologies, particularly for long-term, individual-specific studies on small model organisms[26-28]. For example, measuring a large population's behavioral declines with age can be difficult – especially if single-animal resolution is required. It involves culturing each animal in a separate location and manually measuring behavior at different time points. In contrast, automated microfluidic-based platforms[26] have proven to significantly reduce the labor by culturing the animals and tracking their individual behaviors across the entire lifespan. Yet, despite these advancements, robust transcriptome-level gene expression studies based on these engineering/method-based improvements have not been demonstrated. In this perspective, we aim to highlight where the engineering field will need to focus and adapt to help answer these larger, underlying questions in biology that are emerging. One of the next major advancements in biology will depend on developing robust, efficient methods that allow for gene expression quantification with cellular- to tissue-specificity and inter-individuality.

What are significant limitations to the current tools that measure gene expression for single-animal studies?

Gene expression studies on larger model organisms, such as mice and rats, have revealed countless biological insights. However, these investigations typically have relatively low throughput (*i.e.* on the order of tens of animals) due to practical restraints (*e.g.* labor, cost, time, etc.). To study more subtle details of gene expression or to perform experiments that require large populations of animals, such as forward genetic screening, it is much more feasible in smaller models. For example, to study the entire lifespan of a rat could take 2-3 years; in contrast, along with being much lower in cost, the microscopic nematode, *C. elegans,* only lives for about 20 days[29]. While we acknowledge that larger organism-based studies may be more advantageous for many applications (*e.g.* mammalian-specific diseases), in this perspective, we aim to focus on smaller model organisms (*e.g. Drosophila melanogaster*, *Danio rerio* (*i.e.* zebrafish), *C. elegans*, etc.) where gene expressions studies can be conducted in much larger populations (*i.e.* on the order of tens to hundreds to thousands of animals), and large-scale tissue specific investigations are more accessible. The current popular approaches to quantify

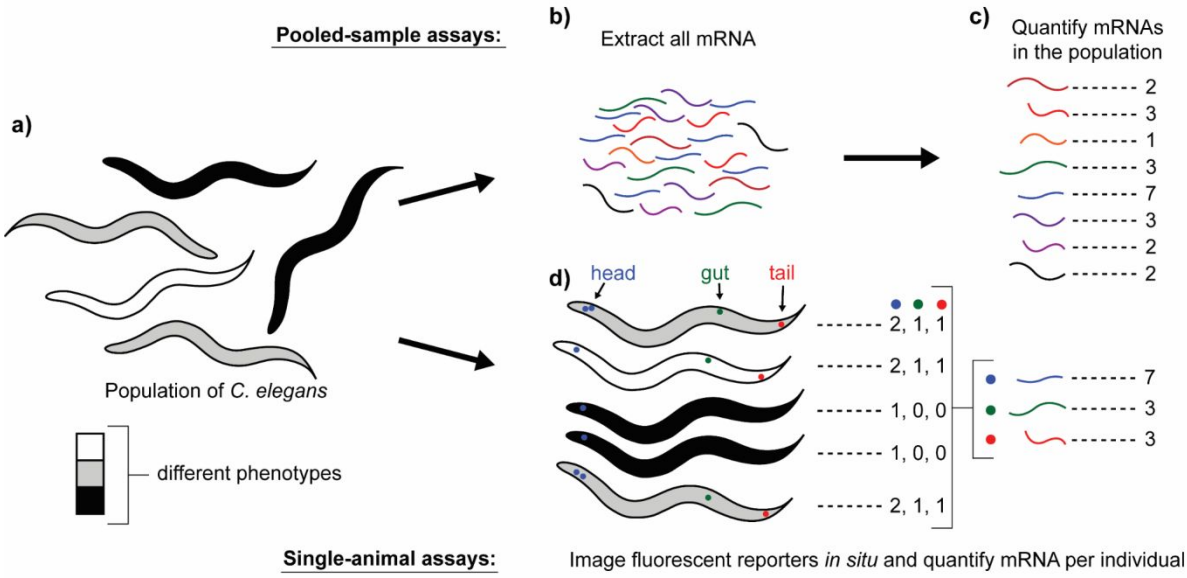


Figure 1. Conceptual model for gene expression quantification assays. In this example, we want to quantify the gene expression of (a) a population of C. elegans with different phenotypes indicated by the shading (white, grey, and black). To perform a pooled-sample assay, (b) the mRNA is extracted from the population of animals and (c) quantified. For single-animal assays, (d) gene expression can be measured within each animal with high spatial resolution. Each color represents a different mRNA sequence, and the numbers represent units of gene expression (A.U.) quantified.

gene expression within these organisms can be divided into two categories: pooled-sample techniques and single-animal techniques (Fig. 1, Table 1).

Pooled-sample techniques that measure gene expression include cDNA microarrays, RNA-seq, and scRNA-seq, which have been reviewed in depth[8, 25, 30, 31] (Table 1). Although each of these tools work differently, the overall pipeline and results are similar. Briefly, the total mRNA is extracted from the sample (Fig. 1a,b), and through different mechanisms, the mRNA is quantified (Fig 1c). cDNA microarrays were one of the first methods to study gene expression on a transcriptome level. With these first investigations in model organisms, such as in flies[32, 33] and worms[34], we began to understand how the gene expression networks and patterns can change due to perturbations, such as aging or genetic mutations. The next major advancement in assays was RNA-seq. Rather than profiling predefined transcripts and genes via hybridization in microarrays, RNA-seq can fully sequence and quantify each mRNA species of the sample. Although these tools can measure the entire transcriptome, the mRNA extraction and processing require the entire population of samples to be pooled together[30, 31]. This loses inter-individuality (*i.e.* we do not know which cell or animal the transcripts are being measured); further, while cellular identity could be inferred for cells with characteristic expression profiles, calling cell identities are not precise and thus, the spatial information of gene expression is not precise (Fig. 1c). Together, while these key methods give a rough indication of which genes are important to a particular process, spatial, temporal, and inter-relational details may be missing. This lack of information can prevent deeper insights into the biological processes.

In order to localize gene expression to specific tissues, these methods have been performed on dissected organs or body parts[2, 32, 45], which require expertise and intensive manual labor. While these studies can quantify gene expression in each tissue, they cannot preserve any individuality. The samples are still pooled together; the information quantified is averaged over the population; importantly, they still lack cellular specificity. To retain inter-individuality, RNA-seq has been adapted to single worms[35]; however, the tissue- and cellular-specificity are still lost.

One strategy to overcome this and measure gene expression with high spatial resolution is to capture the transcripts *in situ* and then perform sequencing *ex situ*[7]. This type of approach was first introduced as "spatial transcriptomics" and demonstrated on thin tissue slices[46]. Briefly, fixed tissue samples are annealed onto glass slides that have barcoded reverse transcriptase primers in a known pattern. The transcripts are sequenced and, since the location of each different bar code is known, computational reconstruction can reveal the spatial distribution of the transcript[46]. Recently, there have been advances to the technique, such as Slide-seq[47]. Here, the barcodes are attached to 10 µm beads rather than printed on a glass slide. While this type of approach can measure genome-wide expression at high spatial resolution[47], this has only been demonstrated on thin tissue slices (~10 µm thick); it may be difficult to scale to whole animals, and certainly not at population levels.

Perhaps the most advanced assay currently available to measure gene expression is scRNA-seq[17]. Briefly, the cells of the sample, such as a population of animals, are isolated and individually sequenced. While this can reveal very high-detailed information, there are some notable caveats. Along with the need for large population sizes (~1,000s to ~10,000s of animals)[5, 48] and lack of individual-level quantification, scRNA-seq can only infer each cell's identity based on its expression profile[37]. For example, there may be a panel of known neuronally expressed genes for a particular animal; cells that have

Table 1. Generalized comparison of pooled-sample and single-animal techniques

	Technique	Single animal	Spatial resolution	Gene throughput	Ease-of-use	Other notes
Pooled-sample Techniques	**cDNA Microarray**[30, 31, 34]	No	Whole animal to dissected body parts	Transcriptome level	Complex manual handling	Profiles pre-defined transcripts
	RNA-seq[8, 13, 30]	No*	Whole animal to dissected body parts	Transcriptome level	Complex manual handling	*RNA-seq has been adapted to single worms[35]
	scRNA-seq[5, 8, 36, 37]	No	Inferred/predicted cells	Transcriptome level	Complex manual handling	Requires large populations (~1,000 to 10,000 animals)
Single-animal Techniques	**Fluorescent Transcriptional Reporter**[38-40]	Yes	Cell to subcellular	Typically, 2-3 genes	Strain engineering and manual handling	Gene throughput limited by number of resolvable fluorophores
	smFISH[6, 41, 42]	Yes	Subcellular	Typically, 2-3 genes	Probe design and manual handling	Gene throughput can be increased with alternative strategies[43, 44], but these have not been achieved in whole animals

higher expression of these genes will thus more likely be from a neuron. In order to be accurate, these methods rely on existing spatial expression data for an array of panel genes[49]. While larger tissues or cells with well-defined transcriptomes may have accurate predictions, smaller tissues or more ambiguous cells may be incorrectly labelled. This level of noise is perhaps further amplified as scRNA-seq is unable to reliably measure or detect transcripts in low abundance[48]. In addition, the sample processing steps are nontrivial and lose many of the cells, or worse, cell types[5, 37]. These technical limitations can complicate data analysis and overlook more subtle details of gene expression, such as the natural biological variation in the system[36, 50].

While scRNA-seq computational predictions have significantly improved[49, 51], their inferences for cell identification still require validation[52]. There have been improvements in these various pooled-sample techniques, but even the most advanced technologies lack either the ability to quantify gene expression on an individual level or to have true spatial localization. Although this may not be an issue for unicellular models, such as performing scRNA-seq in yeast[11], it cannot be applied to multi-cellular organisms.

For example, we may need to quantify gene expression in a population of *C. elegans*, a well-studied model organism. To quantify the expression, we may start with a group of animals from the same population with naturally different phenotypes, such as activity level (Fig. 1a). After processing and extracting the mRNA of the sample (Fig. 1b), we can quantify the amount of each mRNA in the system (Fig. 1c); however, we lose where in the animal the expression occurred, whether there was animal-to-animal variability, and if there were any correlations between the measured gene expressions and the phenotypes observed. These are major limitations to the types of biological questions we can address.

In contrast, to measure finer details, there are high resolution single-animal techniques such as fluorescent transcriptional reporters[40] and smFISH[41] (Table 1). These methods allow researchers to use fluorescent microscopy to visualize and quantify gene expression with tissue specificity (Fig. 1d). In this case, we might find that the genes are expressed in different locations/tissues (*i.e.* head, gut, and tail) (Fig. 1d). In addition, single-animal resolution enables us to find if there are any correlations between the phenotypes of the animals and their unique gene expression profiles. This would enable us to ask questions, such as how the phenotypic variability is influenced by the gene expression variability. It may be that, among the population of animals, differences in local environments contribute to variability in gene expression and behavior. For example, *daf-7* is a gene that is typically only expressed in one pair of neurons in *C. elegans*; however, when the animals are exposed to pathogenic bacteria, *daf-7* expression occurs in different neurons, and the animals display an avoidance behavior[53]. There are other assays to measure expression on a single-animal or single-cell level such as the cell-based MS2-MCP system[54-56], immunohistochemistry to quantify protein expression, and newer, emerging technologies such as CRISPR-dCas13a[57] to measure mRNA *in vivo*. In this perspective, we chose to focus on these two more popular and commonly used methods in model organisms: fluorescent transcriptional reporters and smFISH. These approaches can be complimentary, and their quantification is based on fluorescent microscopy.

Fluorescent transcriptional reporters allow us to measure gene expression activity in live animals with tissue specificity. Here, a fluorophore, such as GFP, is driven by the promoter fragment of the gene of interest. The genetically modified animal will express the fluorophore; by measuring the fluorescence intensity, we can determine the promoter's activity as well as where it drives expression in the animal[39, 40, 58]. There are variations to this approach such as translational reporters where the fluorophore is directly fused to the protein of interest; this can provide a better measurement on protein expression rather than the transcriptional activity[40]. Further, for transparent transcriptional reporter systems, such as cells[59], yeast[38], and *C. elegans*[40], imaging can be done *in vivo* without much perturbation to the samples, allowing for researchers to track the expression levels in individual samples over time. However, it is important to note that the long half-life of fluorophores (~24 hours)[39, 60, 61] can limit studies dependent on measuring dynamic phenotypes and require higher temporal resolution. To reflect meaningful promoter activity and detect the subtleties of gene expression, fluorescent transcriptional reporters also need to be single copy integrants[62]. Having a single copy ensures that the amount of fluorescence intensity accurately represents the promoter activity. Integrating the exogenous transgene into the genome prevents any mosaicism (*i.e.* integration ensures all cells in the sample have copies of the promoter-driven fluorophore transgene). The major drawback for this method is that it can be difficult and time-consuming to create new strains, which is often the bottleneck for large-scale studies on many genes.

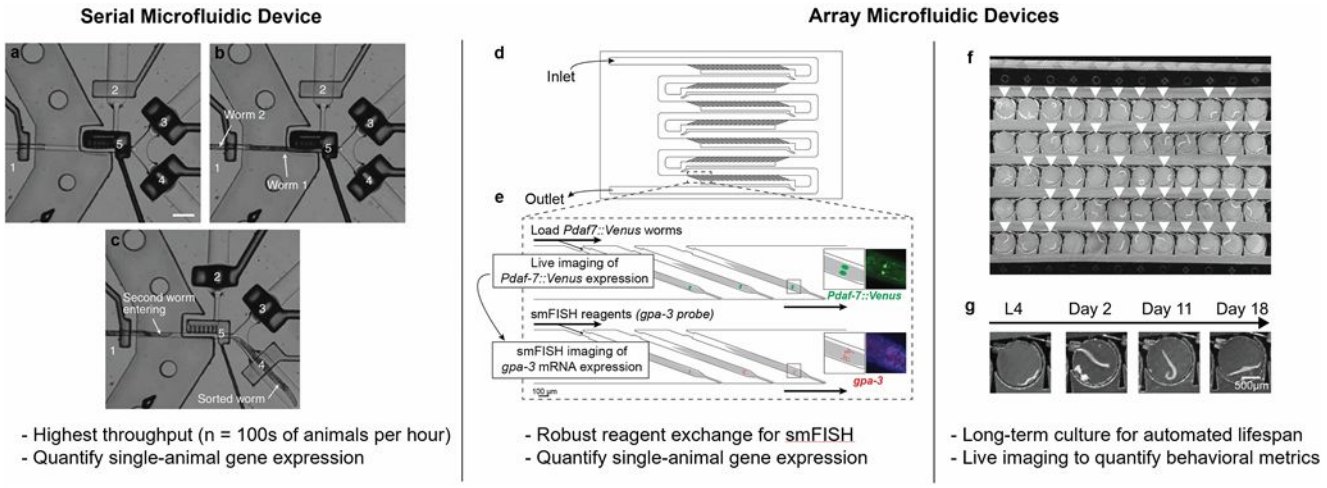


Figure 2. Examples of single-animal microfluidic devices. Typically, devices fall under two categories: (a-c) serial (Adapted from Ref. 94 with permission from Springer Nature) and (d-g) array devices. (a-c) Serial devices can have a much higher sample throughput as they are not constrained by the geometry of the device (*i.e.* number of traps). By controlling a set of on-chip valves, (a) one can prepare the device and imaging location, (b) load a fluorescent transcriptional reporter worm and prevent a second worm from entering, and (c) sort the imaged worm and automatically load the second worm into the detection zone. Scale bar, 100 μm. (d) Microfluidic array devices can geometrically isolate samples for single-animal analysis. (e) This device enables multiple reagent exchanges; this allowed for the measurement of a live fluorescent transcriptional reporter and smFISH to quantify multiple genes within the same animal. (f) Array strategies have also been used for long-term culture. (g) In this demonstration, animals are loaded into the device and automated imaging allows one to measure behavior and healthspan metrics across the lifespan of multiple animals. Adapted from Ref. 99 (d,e) and Ref. 26 (f,g) with permission from the Royal Society of Chemistry and Springer Nature, respectively.

Another single-animal technique to measure *in situ* gene expression is smFISH. In this approach, gene-specific, fluorescently labelled probes are delivered into a fixed and permeabilized sample. In contrast to fluorescent transcriptional reporters, this method does not require any genetic manipulations. This makes smFISH particularly attractive for many genomic-based studies that require non-transgenic animals (*e.g.* studying wildtype animals, genome-wide association studies, etc.). In smFISH, a gene-specific probe set consists of 20-40 fluorescently labelled short nucleic acids (each ~25 nts); each individual probe within the set has a different sequence that binds specifically to the target mRNA. To ensure specificity and prevent false-positive signals from non-specific binding, a fluorescent punctum only becomes resolvable when 20-30 probes hybridize onto the same mRNA molecule due to the high local concentration of the probes[41]. This approach allows us to visualize and measure individual mRNA molecules within the fixed sample with sub-cellular spatial resolution. Along with quantifying the amount of gene expression, its use in model organisms is often to localize gene expression and evaluate spatial organization[63, 64]. Despite its advantages, there are also several drawbacks to this method. Along with long incubation times (*i.e.* many hours to days) for different reagents, durations, and temperatures, it requires sample fixation and permeabilization, thus limiting its use as an endpoint assay.

While both single-animal techniques offer much more detailed information and allow us to study more subtle details of gene expression, they lack gene- and sample-throughput. These techniques are based on fluorescent microscopy; this limits the number of genes studied per animal to the number of spectrally resolvable fluorophores on an imaging setup (typically 2-3). Another shortcoming is that these methods require slow, manual handling and imaging of each sample. Increasingly, transcriptome-level studies using pooled-sample techniques are inspiring questions in biology that depend on single-animal resolution. These shortcomings to these methods have not yet been completely addressed, and thus present engineering opportunities.

How can we improve single-animal techniques?

To improve upon the single-animal techniques introduced above, there are two separate approaches: reagent-based improvements and sample-handling improvements. Reagent-based improvements refer to advances in reagents for currently available techniques and assays, including new fluorophores or strategies for assaying larger numbers of genes. Sample-handing improvements include methods for robust high-throughput imaging, reagent delivery, and long-term culturing. Advancements in both fields can improve the feasibility and accessibility for single-animal based gene-expression studies, as well as the types of questions we can answer.

In the past few years, there have been notable advancements in reagents that increase the sensitivity of expression measurements, improve temporal resolution, and increase gene throughput (*i.e.* number of genes studied per sample). For fluorescent transcriptional reporters, a large number of new, brighter fluorophores have been reported (*e.g.* mScarlet[65] and mNeonGreen[66]), which can be especially useful for genes with naturally low levels of expression or experiments that require higher precision. Another method to improve a fluorophore's expression is species-specific codon optimization[67], which enables more stable expression and stronger fluorescent signalling. There are also a number of sequences that can be tagged onto the fluorophore to improve different types of resolutions. For better spatial resolution, there are sequences to

promote nuclear[68, 69], cytosolic[70], and/or membrane-tagged localization[71, 72]. Each has their own advantages depending on the situation. For example, studies examining neuronal connections may require membrane-tagged fluorophores to enhance the fluorescence intensity in the axons and dendrites[73]. For tighter temporal resolution, destabilizing the fluorophore can reduce its half-life by an order of magnitude[39, 73], making them particularly useful for measuring dynamic processes such as transitions in the cell cycle[74].

Similarly, there have been many reagent-based or biochemistry/biotechnology-based improvements to smFISH. Many of these methods have been recently reviewed in depth[6, 7]. Briefly, to amplify the fluorescent puncta's signal and resolution, there are ways to increase the number of fluorophores bound to the target mRNA. Amplified signals are important for many applications; for instance, tissue samples have a significantly higher autofluorescence compared to cell culture, and thus require higher signals to differentiate from the background[75]. In traditional smFISH, each primary probe has one bound fluorophore. While this may be sufficient, some applications require stronger signals. By simply increasing each probe to have two fluorophores, the fluorescent signal is roughly doubled[76]. There are also methods to increase the signal by at least an order of magnitude. These techniques typically use fluorescently labelled secondary probes; these bind to sequence-specific primary probes and amplifiers, which directly bind to the target mRNA[76-78]. Other strategies[79], including hybridization chain reaction (HCR)[75] and padlock probes[80], can exponentially amplify the signals 10- to 100-fold[6]. Each of these methods can improve the fluorescent signals, but importantly, they also increase the complexity of the experiments with additional steps and/or reagents. Some applications may have different requirements, and this trade-off is important to consider. A potential concern with having these amplified signals is spatial resolution. Since each fluorescent punctum corresponds to an individual mRNA, it can be difficult to distinguish each punctum from one another if the target mRNA is in high abundance or has a high signal that create overlaps (*i.e.* the puncta are too close to visually separate). To address this, smFISH has been used in conjunction with expansion microscopy to achieve nanoscale imaging[81, 82].

Another major improvement in smFISH-based technologies is the use of stripping reagents that enable multiple cycles or rounds of smFISH within the same sample[24, 42]. When used in combination with multiplexing and coding strategies, the number of genes measured exponentially increases with each round of smFISH and can even span the transcriptome[43, 44]. To implement multiplexing strategies, one needs to track the same transcript across multiple rounds of smFISH. This is accomplished by mounting the fixed sample onto a glass slide to prevent any movement. However, this has only been achieved in samples of cells and tissue slices. Due to the larger number of reagent exchanges, high transport requirements, and the need for continuous cell and transcript identification across multiple rounds of smFISH, this has not yet been achieved in whole organisms and these current methods are perhaps not scalable to large populations. Microfluidic-based devices, however, have been demonstrated to enhance molecular transport and enable multi-cycle smFISH (*i.e.* 2 rounds of hybridization)[83] in *C. elegans*. This could potentially be further developed for multi-gene level studies (*i.e.* ~10s of genes). Each round of hybridization can measure 2-3 genes (*i.e.* number of spectrally resolvable fluorophores). By stripping these probes and re-hybridizing the same sample, it would be possible to study another set of 2-3 genes; this strategy requires continuous animal identification to relate the genes per animal from different hybridization rounds. While multiplexing and coding strategies can exponentially increase the number of genes studied per round, these techniques require continuously tracking the identity of each cell and transcript. While microfluidics or even image-processing techniques may be able to maintain this type of information, it has still not been robustly demonstrated. Flowing different reagents into a microfluidic device can often move a sample, and even slight shifts in location or orientation can result in a loss of cell or transcript identity.

Microfluidic technologies have made notable sample handling improvements, particularly for smaller model organisms such as *Drosophila*[84-86], *Danio renio*[87, 88], *C. elegans*[26, 27, 89-91], and more[92, 93]. Due to each organism's unique properties, there are differences in the types of devices and their respective capabilities. For example, *C. elegans* can be cultured in liquid; this perhaps makes long-term culture in microfluidic devices easier to adapt compared to adult *Drosophila*. Collectively, when considering microfluidics' impact on model organism-based studies, there are improvements for high-throughput imaging, more efficient reagent delivery, and well-controlled long-term culturing. For these single-animal techniques, imaging is typically a bottleneck for sample throughput. Traditionally, for both fluorescent transcriptional reporters and smFISH, animals are collected and placed onto a glass slide and imaged; the whole process for imaging a single sample can take ~5-10 minutes. Although simply imaging a population at a single time point can be relatively easy, this task becomes much more burdensome if the investigation requires more precise details, such as the identity of each animal across multiple assays, or large population studies. For example, one may want to ask why a certain behavior differs in either response or prevalence among an isogenic population[21]. A possible explanation is that the natural stochasticity of gene expression plays a role in the variability of behavior between the individuals. In order to test this, one could measure the behavior of a fluorescent transcriptional reporter and then image it to quantify the gene expression on a per animal basis. While this can be done manually with significant time and labor (*i.e.* on the order of tens of hours for 100 animals), microfluidic technologies can offer much more efficient, high-throughput imaging. This is typically achieved through two different strategies: serial and array devices (Fig. 2).

In the serial strategy, animals are loaded into the microfluidic device and imaged one at a time (Fig. 2 a-c). In this example[94] fluorescent transcriptional reporters are first loaded into the device and positioned by operating a series of on-chip valves (Fig. 2a,b). Animals, such as *C. elegans*, typically have natural locomotion which can making imaging difficult. There are different techniques for different animals to reduce movement, and this device implements a cooling channel to immobilize the worm during imaging. By activating different valves, the animal is flushed out of the device

(Fig. 2c) and the next one is automatically loaded. While device design and fabrication for these serial devices may be challenging, they are capable of the highest sample throughput. With a high level of control through a combination of on-chip valves, this has been demonstrated to be an effective method for high-throughput imaging (*i.e.* hundreds of animals per hour[94])[91, 95, 96] and could be adapted for animal retrieval post-imaging. By improving sample-throughput, these serial devices may also be able to address gene-throughput. Although each image or animal sample may only be used to measure the number of genes equal to the number of spectrally resolvable fluorophores, a microfluidic pipeline could easily measure multiple samples. For example, one may be interested in studying a gene network comprised of ten genes. Following traditional techniques, one may measure the expression of ten fluorescent reporter strains, each a reporter for a different gene. It could take up to hundreds of hours to image 100 animals per strain. In contrast, a serial microfluidic device can image all ten strains within ten hours based on the reported sample-throughput.

For array devices (Fig. 2d-g), animals are loaded into separate traps or chambers[26, 85, 97, 98]; typically, each chamber holds an individual animal. While array strategies' sample sizes may be limited by the number of slots in the device, array devices can easily maintain the identity of each sample through their geometric constraints. This can be particularly advantageous if multiple assays on the same animal are needed for a study. For example, one may be interested in a live phenotype and its associated gene expression. This array-based microfluidic device (Fig. 2d) demonstrated this by first imaging a live fluorescent transcriptional reporter and then performing smFISH to measure two different genes within the same animal (Fig. 2e)[99]. There are numerous variations of each strategy, and all have shown substantial improvements to imaging throughput when compared to the off-chip methods. Another key advantage to array devices is that, depending on the size of the chambers, it may be possible to monitor behavior on-chip as well (Fig. 2f,g). In this example[26], researchers designed a microfluidic device for long-term culture of *C. elegans* (Fig. 2f). By implementing an automated imaging set-up, they demonstrated the ability to measure behavioral phenotypes and healthspan metrics within individuals throughout the lifespan of the animal (Fig. 2g). Along with imaging, microfluidic devices have also demonstrated improved reagent delivery[99, 100], which is essential for many bioassays including smFISH (Fig. 2e). There are also opportunities to incorporate other phenomena or mechanisms, such as electrokinetics, to increase transport efficiency[83]. While there have been numerous improvements that can make single-animal assays more feasible and accessible, there is still no demonstration of robust, transcriptome-level or even multi-gene (*i.e.* tens to hundreds) method demonstrated on whole single organisms.

Looking forward

Pooled-sample assays have enabled biologists to study new transcriptome-level explorations. As we are learning more and more about the broad impact of gene expression dynamics and patterns on living systems, we are beginning to focus our biological questions that ultimately depend on single-organism resolution. In this perspective, we highlighted two major categories of advancements to single-animal assays: (1) the robustness of the reagents and (2) the tools to improve sample handling and throughput. Although reagent-based improvements have substantially increased the broad use and impact of these single-animal techniques, the other major bottleneck is still present – sample handling. In contrast to pooled-sample techniques, the total number manually assayed in traditional single-animal techniques is typically much lower, due to the need to physically manipulate and image each individual, reducing the power of the assay to detect differences between groups. Even with the unique advantages of these single-animal techniques, the feasibility and uses of single-animal assays are still limited, especially when compared to pooled-sample assays. There is an increasing need to improve our single-animal based assays, and thus, this presents new, demanding opportunities where engineering new methods can make meaningful impacts.

Microfluidic technologies are perhaps one of the most well-suited candidates capable of improving single-animal gene expression studies. There are many devices that increase the imaging throughput for different animals, and it has been demonstrated that hundreds of animals can be imaged within a few hours[94]. This sample-throughput achieved is on-par with pooled-sample techniques, such as RNA-seq, while maintaining the spatial expression information on an individual level. With slight modifications to the protocols, other serial-imaging devices may incorporate fluorescent transcriptional reporters of different animals to measure the gene expression profiles with spatial- and individual-level resolution. With the capacity for high-throughput studies, one can quickly image multiple different transcriptional reporters to examine multiple genes. Further, there are a number of micro-scale, long-term culturing devices that are primarily used to measure behavioral phenotypes[26, 101-105]. While there are findings that demonstrate behavior is tied to gene expression, such as in studies that compare behaviors between mutant and wildtype animals, it may be more difficult to find more subtle correlations within a single population. To measure this relationship, one might incorporate fluorescent imaging to a microfluidic culturing system in order to measure the gene expression of a fluorescent transcriptional reporter while recording its behavior. Gathering this information simultaneously within a single population may reveal insights into how natural variability of gene expression may arise and its influences on behavior[106].

Perhaps the largest disadvantage to these single-animal assays is the number of genes feasible to characterize in each experiment. The need for fluorescent imaging limits the number of genes studied per sample to the number of spectrally resolvable fluorophores. It has been demonstrated that smFISH multiplexing strategies can study the transcriptome of cells and tissues samples[43, 44] – but how can we adapt this for whole animals? Microfluidic arrays offer a potential solution. By having geometric constraints, one can confidently measure expressions of an individual sample. Incorporating multicycle smFISH[83] (*i.e.* hybridizing a probe set, stripping the probe

set, hybridizing a different probe set, etc.) or multiplexing techniques (which microfluidics is suitable to do), we may be able to study multiple genes with sub-cellular spatial resolution in single animals. While these advancements require many technical considerations, this may be a method to overcome the gene throughput limitations of smFISH.

Adapting and advancing microfluidic-based technologies can have many technical hurdles and addressing them can improve a platform's ease-of-use, accessibility, and overall impact. One potential downside for many existing devices is the need for off-chip components, such as a syringe pump or pressure regulator to drive fluid into the microfluidic device or operate on-chip valves. This requirement may limit the accessibility and broad use of microfluidic devices. In addition, interfacing macro- and micro-scaled parts can have many challenges. For example, many macro-scaled parts, such as syringes or tubing, handle large volumes relative to the microfluidic device. This can be detrimental for studies that require expensive reagents or precise exchange of small volumes (nano- to microliter-scale). Additionally, while microfluidics can perform robust reagent delivery, the samples within the device might experience high shear forces due to the fluid flow, which may affect the mechanical integrity of the samples. It is also important to note that many animal-based microfluidic devices are designed for embryos, larvae, and *C. elegans*. While microfluidics can offer a unique level of control, they can be limited by the size or complexity of the animal, such as the types of natural behaviors. However, it may be possible to adapt overall strategies of microfluidics to larger mesofluidic devices[107] or droplet/multi-well formats[108] for animal culture. By overcoming these issues and delivering robust handling, we envision that microfluidic technologies can play a major role in advancing biology.

In this perspective, we aimed to outline promising opportunities for engineers to create new methods and approaches that address the sample-handling limitations of our current techniques. At present, we rely on pooled-sample assays to understand how gene expression may change or react to a perturbation (*e.g.* developmental stage, age, temperature, food availability, etc.). Armed with better, cheaper, more robust, more parallel, high-resolution techniques for single-cell or single-animal gene-expression analyses, we envision an expanding frontier of biological inquiries, addressing questions such as how phenotypic variation contributes to the developmental process or to the declines of aging, how tissues respond differently to stimuli, whether certain tissues are more susceptible to stress-induced decline, and beyond.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

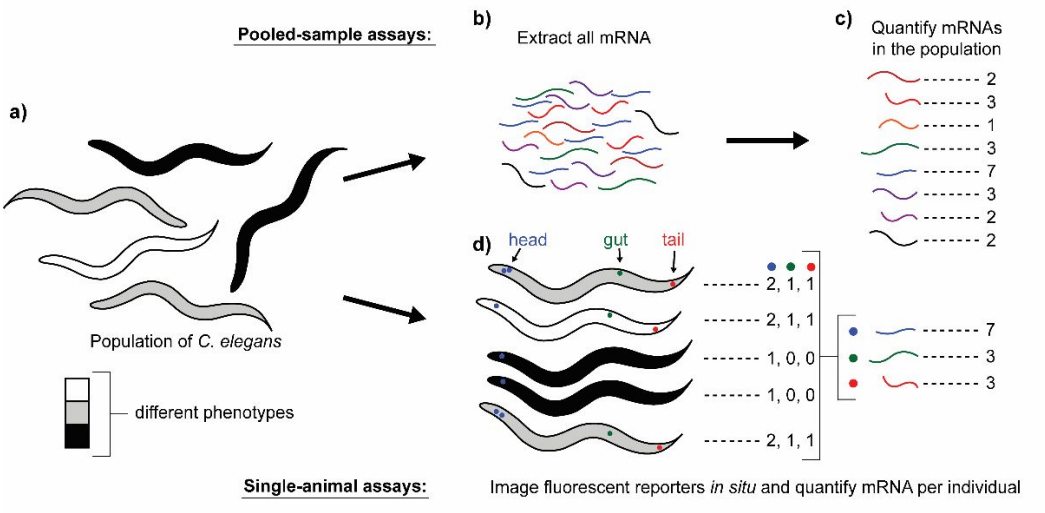
We would like to acknowledge funding from the National Institutes of Health (R01GM088333, R01AG056436, R01NS115484, R21DC015652, R21NS117066, R21NS117067), National Science Foundation (DBI1707401 and DMS1764406) and Simons Foundation/SFARI 594594 to HL. JW would like to acknowledge support from the National Science Foundation Graduate Research Fellowship (DGE-1650044).

References

1. W. Zhao, X. Ji, F. Zhang, L. Li and L. Ma, *Molecules*, 2012, **17**, 6196-6236.
2. S. Ma, S. Sun, L. Geng, M. Song, W. Wang, Y. Ye, Q. Ji, Z. Zou, S. Wang, X. He, W. Li, C. R. Esteban, X. Long, G. Guo, P. Chan, Q. Zhou, J. C. I. Belmonte, W. Zhang, J. Qu and G.-H. Liu, *Cell*, 2020, **180**, 984-1001.e1022.
3. R. K. Saiki, D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich, *Science*, 1988, **239**, 487.
4. 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.
5. Aleksandra A. Kolodziejczyk, J. K. Kim, V. Svensson, John C. Marioni and Sarah A. Teichmann, *Mol Cell*, 2015, **58**, 610-620.
6. X. Pichon, M. Lagha, F. Mueller and E. Bertrand, *Mol Cell*, 2018, **71**, 468-480.
7. M. Asp, J. Bergenstråhle and J. Lundeberg, *BioEssays*, 2020, **n/a**, 1900221.
8. A. Kulkarni, A. G. Anderson, D. P. Merullo and G. Konopka, *Curr. Opin. Biotechnol.*, 2019, **58**, 129-136.
9. A. P. Gasch, F. B. Yu, J. Hose, L. E. Escalante, M. Place, R. Bacher, J. Kanbar, D. Ciobanu, L. Sandor, I. V. Grigoriev, C. Kendziorski, S. R. Quake and M. N. McClean, *PLoS Biol*, 2017, **15**, e2004050.
10. A. Jariani, L. Vermeersch, B. Cerulus, G. Perez-Samper, K. Voordeckers, T. Van Brussel, B. Thienpont, D. Lambrechts and K. J. Verstrepen, *eLife*, 2020, **9**, e55320.
11. P. Liu, R. Song, G. L. Elison, W. Peng and M. Acar, *Nat. Commun.*, 2017, **8**, 680.
12. M. Ximerakis, S. L. Lipnick, B. T. Innes, S. K. Simmons, X. Adiconis, D. Dionne, B. A. Mayweather, L. Nguyen, Z. Niziolek, C. Ozek, V. L. Butty, R. Isserlin, S. M. Buchanan, S. S. Levine, A. Regev, G. D. Bader, J. Z. Levin and L. L. Rubin, *Nat. Neurosci.*, 2019, **22**, 1696-1708.
13. S. H. Wood, T. Craig, Y. Li, B. Merry and J. P. de Magalhães, *AGE*, 2013, **35**, 763-776.
14. D. Bazopoulou, D. Knoefler, Y. Zheng, K. Ulrich, B. J. Oleson, L. Xie, M. Kim, A. Kaufmann, Y.-T. Lee, Y. Dou, Y. Chen, S. Quan and U. Jakob, *Nature*, 2019, **576**, 301-305.
15. S. Gelino, J. T. Chang, C. Kumsta, X. She, A. Davis, C. Nguyen, S. Panowski and M. Hansen, *PLoS genetics*, 2016, **12**, e1006135-e1006135.
16. B. Zhang, J. Gong, W. Zhang, R. Xiao, J. Liu and X. Z. S. Xu, *Genes Dev*, 2018, **32**, 258-270.
17. J. Cao, J. S. Packer, V. Ramani, D. A. Cusanovich, C. Huynh, R. Daza, X. Qiu, C. Lee, S. N. Furlan, F. J. Steemers, A. Adey, R. H. Waterston, C. Trapnell and J. Shendure, *Science*, 2017, **357**, 661.
18. B. A. Benayoun, E. A. Pollina, P. P. Singh, S. Mahmoudi, I. Harel, K. M. Casey, B. W. Dulken, A. Kundaje and A. Brunet, *Genome Res*, 2019, **29**, 697-709.
19. B. W. Dulken, M. T. Buckley, P. Navarro Negredo, N. Saligrama, R. Cayrol, D. S. Leeman, B. M. George, S. C.

Boutet, K. Hebestreit, J. V. Pluvinage, T. Wyss-Coray, I. L. Weissman, H. Vogel, M. M. Davis and A. Brunet, *Nature*, 2019, **571**, 205-210.
20. Z. Pincus, T. Smith-Vikos and F. J. Slack, *PLoS Genet.*, 2011, **7**, e1002306.
21. S. Stern, C. Kirst and C. I. Bargmann, *Cell*, 2017, **171**, 1649-1662.e1610.
22. S. Ben-Moshe and S. Itzkovitz, *Nat. Rev. Gastroenterol.*, 2019, **16**, 395-410.
23. K. Joshi, M. R. de Massy, M. Ismail, J. L. Reading, I. Uddin, A. Woolston, E. Hatipoglu, T. Oakes, R. Rosenthal, T. Peacock, T. Ronel, M. Noursadeghi, V. Turati, A. J. S. Furness, A. Georgiou, Y. N. S. Wong, A. Ben Aissa, M. W. Sunderland, M. Jamal-Hanjani, S. Veeriah, N. J. Birkbak, G. A. Wilson, C. T. Hiley, E. Ghorani, J. A. Guerra-Assunção, J. Herrero, T. Enver, S. R. Hadrup, A. Hackshaw, K. S. Peggs, N. McGranahan, C. Swanton, S. A. Quezada and B. Chain, *Nat Med*, 2019, **25**, 1549-1559.
24. T. Masuda, R. Sankowski, O. Staszewski, C. Böttcher, L. Amann, Sagar, C. Scheiwe, S. Nessler, P. Kunz, G. van Loo, V. A. Coenen, P. C. Reinacher, A. Michel, U. Sure, R. Gold, D. Grün, J. Priller, C. Stadelmann and M. Prinz, *Nature*, 2019, **566**, 388-392.
25. J. Liao, X. Lu, X. Shao, L. Zhu and X. Fan, *Trends Biotechnol.*, DOI: 10.1016/j.tibtech.2020.05.006.
26. K. N. Le, M. Zhan, Y. Cho, J. Wan, D. S. Patel and H. Lu, *Commun. Biol.*, 2020, **3**, 297.
27. H. B. Atakan, R. Xiang, M. Cornaglia, L. Mouchiroud, E. Katsyuba, J. Auwerx and M. A. M. Gijs, *Sci. Rep.*, 2019, **9**, 14340.
28. W. Keil, L. M. Kutscher, S. Shaham and E. D. Siggia, *Dev. Cell*, 2017, **40**, 202-214.
29. C. J. Kenyon, *Nature*, 2010, **464**, 504-512.
30. S. Zhao, W.-P. Fung-Leung, A. Bittner, K. Ngo and X. Liu, *PLOS ONE*, 2014, **9**, e78644.
31. K. J. Mantione, R. M. Kream, H. Kuzelova, R. Ptacek, J. Raboch, J. M. Samuel and G. B. Stefano, *Med Sci Monit Basic Res*, 2014, **20**, 138-142.
32. F. Girardot, C. Lasbleiz, V. Monnier and H. Tricoire, *BMC Genomics*, 2006, **7**, 69.
33. M. Zhan, H. Yamaza, Y. Sun, J. Sinclair, H. Li and S. Zou, *Genome Res*, 2007, **17**, 1236-1243.
34. T. R. Golden and S. Melov, *Aging Cell*, 2004, **3**, 111-124.
35. L. Serra, D. Z. Chang, M. Macchietto, K. Williams, R. Murad, D. Lu, A. R. Dillman and A. Mortazavi, *Bio-protocol*, 2018, **8**, e2729.
36. P. Brennecke, S. Anders, J. K. Kim, A. A. Kołodziejczyk, X. Zhang, V. Proserpio, B. Baying, V. Benes, S. A. Teichmann, J. C. Marioni and M. G. Heisler, *Nat. Methods*, 2013, **10**, 1093-1095.
37. S. Liu and C. Trapnell, *F1000Res*, 2016, **5**, F1000 Faculty Rev-1182.
38. S. Wildt and U. Deuschle, *Nat. Biotechnol.*, 1999, **17**, 1175-1178.
39. L. He, R. Binari, J. Huang, J. Falo-Sanjuan and N. Perrimon, *eLife*, 2019, **8**, e46181.
40. T. Boulin, J. F. Etchberger and O. Hobert, Reporter gene fusions, *Wormbook,* The C. elegans Research Community, 2006, DOI: 10.1895/wormbook.1.106.1
41. A. Raj, P. van den Bogaard, S. A. Rifkin, A. van Oudenaarden and S. Tyagi, *Nat. Methods*, 2008, **5**, 877-879.
42. K. H. Chen, A. N. Boettiger, J. R. Moffitt, S. Wang and X. Zhuang, *Science*, 2015, **348**, aaa6090.
43. C.-H. L. Eng, M. Lawson, Q. Zhu, R. Dries, N. Koulena, Y. Takei, J. Yun, C. Cronin, C. Karp, G.-C. Yuan and L. Cai, *Nature*, 2019, **568**, 235-239.
44. C. Xia, J. Fan, G. Emanuel, J. Hao and X. Zhuang, *PNAS*, 2019, **116**, 19490.
45. R. Pacifico, C. M. MacMullen, E. Walkinshaw, X. Zhang and R. L. Davis, *PloS One*, 2018, **13**, e0209405-e0209405.
46. P. L. Ståhl, F. Salmén, S. Vickovic, A. Lundmark, J. F. Navarro, J. Magnusson, S. Giacomello, M. Asp, J. O. Westholm, M. Huss, A. Mollbrink, S. Linnarsson, S. Codeluppi, Å. Borg, F. Pontén, P. I. Costea, P. Sahlén, J. Mulder, O. Bergmann, J. Lundeberg and J. Frisén, *Science*, 2016, **353**, 78.
47. S. G. Rodriques, R. R. Stickels, A. Goeva, C. A. Martin, E. Murray, C. R. Vanderburg, J. Welch, L. M. Chen, F. Chen and E. Z. Macosko, *Science*, 2019, **363**, 1463.
48. S. Islam, A. Zeisel, S. Joost, G. La Manno, P. Zajac, M. Kasper, P. Lönnerberg and S. Linnarsson, *Nat. Methods*, 2014, **11**, 163-166.
49. R. Satija, J. A. Farrell, D. Gennert, A. F. Schier and A. Regev, *Nat. Biotechnol.*, 2015, **33**, 495-502.
50. G. K. Marinov, B. A. Williams, K. McCue, G. P. Schroth, J. Gertz, R. M. Myers and B. J. Wold, *Genome Res*, 2014, **24**, 496-510.
51. K. Achim, J.-B. Pettit, L. R. Saraiva, D. Gavriouchkina, T. Larsson, D. Arendt and J. C. Marioni, *Nat. Biotechnol.*, 2015, **33**, 503-509.
52. A. K. Shalek, R. Satija, J. Shuga, J. J. Trombetta, D. Gennert, D. Lu, P. Chen, R. S. Gertner, J. T. Gaublomme, N. Yosef, S. Schwartz, B. Fowler, S. Weaver, J. Wang, X. Wang, R. Ding, R. Raychowdhury, N. Friedman, N. Hacohen, H. Park, A. P. May and A. Regev, *Nature*, 2014, **510**, 363-369.
53. Joshua D. Meisel, O. Panda, P. Mahanti, Frank C. Schroeder and Dennis H. Kim, *Cell*, 2014, **159**, 267-280.
54. M. Vera, E. Tutucci and R. H. Singer, in *Imaging Gene Expression: Methods and Protocols*, ed. Y. Shav-Tal, Springer New York, New York, NY, 2019, DOI: 10.1007/978-1-4939-9674-2_1, pp. 3-20.
55. E. Tutucci, M. Vera, J. Biswas, J. Garcia, R. Parker and R. H. Singer, *Nat. Methods*, 2018, **15**, 81-89.
56. X. Pichon, M.-C. Robert, E. Bertrand, R. H. Singer and E. Tutucci, in *RNA Tagging: Methods and Protocols*, ed. M. Heinlein, Springer US, New York, NY, 2020, DOI: 10.1007/978-1-0716-0712-1_7, pp. 121-144.
57. O. O. Abudayyeh, J. S. Gootenberg, P. Essletzbichler, S. Han, J. Joung, J. J. Belanto, V. Verdine, D. B. T. Cox, M. J. Kellner, A. Regev, E. S. Lander, D. F. Voytas, A. Y. Ting and F. Zhang, *Nature*, 2017, **550**, 280-284.
58. M. Chalfie, Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher, *Science*, 1994, **263**, 802.
59. N. E. Phillips, A. Mandic, S. Omidi, F. Naef and D. M. Suter, *Nat. Commun.*, 2019, **10**, 1208.
60. X. Li, X. Zhao, Y. Fang, X. Jiang, T. Duong, C. Fan, C. C. Huang and S. R. Kain, *J Biol Chem*, 1998, **273**, 34970-34975.
61. E. L. Snapp, *Trends Cell Biol.*, 2009, **19**, 649-655.
62. C. Frøkjær-Jensen, M. Wayne Davis, C. E. Hopkins, B. J. Newman, J. M. Thummel, S.-P. Olesen, M. Grunnet and E. M. Jorgensen, *Nat. Genet.*, 2008, **40**, 1375-1383.
63. T. Trcek, M. Grosch, A. York, H. Shroff, T. Lionnet and R. Lehmann, *Nat. Commun.*, 2015, **6**, 7962.

64. R. Posner, I. A. Toker, O. Antonova, E. Star, S. Anava, E. Azmon, M. Hendricks, S. Bracha, H. Gingold and O. Rechavi, *Cell*, 2019, **177**, 1814-1826.e1815.
65. D. S. Bindels, L. Haarbosch, L. van Weeren, M. Postma, K. E. Wiese, M. Mastop, S. Aumonier, G. Gotthard, A. Royant, M. A. Hink and T. W. J. Gadella, *Nat. Methods*, 2017, **14**, 53-56.
66. N. C. Shaner, G. G. Lambert, A. Chammas, Y. Ni, P. J. Cranfill, M. A. Baird, B. R. Sell, J. R. Allen, R. N. Day, M. Israelsson, M. W. Davidson and J. Wang, *Nat. Methods*, 2013, **10**, 407-409.
67. G. Hanson and J. Coller, *Nat Rev Mol Cell Biol*, 2018, **19**, 20-30.
68. G. Fujii, R. Tsuchiya, E. Ezoe and S. Hirohashi, *Exp. Cell Res.*, 1999, **251**, 299-306.
69. N. M. Seibel, J. Eljouni, M. M. Nalaskowski and W. Hampe, *Anal Biochem*, 2007, **368**, 95-99.
70. R. Rizzuto, M. Brini, P. Pizzo, M. Murgia and T. Pozzan, *Curr. Biol.*, 1995, **5**, 635-642.
71. K. Moriyoshi, L. J. Richards, C. Akazawa, D. D. M. O'Leary and S. Nakanishi, *Neuron*, 1996, **16**, 255-260.
72. M. S. Rana, X. Wang and A. Banerjee, *Biochemistry*, 2018, **57**, 6741-6751.
73. H. Gehart, J. H. van Es, K. Hamer, J. Beumer, K. Kretzschmar, J. F. Dekkers, A. Rios and H. Clevers, *Cell*, 2019, **176**, 1158-1173.e1116.
74. C. Mateus and S. V. Avery, *Yeast*, 2000, **16**, 1313-1323.
75. S. Shah, E. Lubeck, M. Schwarzkopf, T.-F. He, A. Greenbaum, C. H. Sohn, A. Lignell, H. M. T. Choi, V. Gradinaru, N. A. Pierce and L. Cai, *Development*, 2016, **143**, 2862-2867.
76. N. Tsanov, A. Samacoits, R. Chouaib, A.-M. Traboulsi, T. Gostan, C. Weber, C. Zimmer, K. Zibara, T. Walter, M. Peter, E. Bertrand and F. Mueller, *Nucleic Acids Res*, 2016, **44**, e165-e165.
77. J. R. Sinnamon and K. Czaplinski, *Rna*, 2014, **20**, 260-266.
78. F. Wang, J. Flanagan, N. Su, L.-C. Wang, S. Bui, A. Nielson, X. Wu, H.-T. Vo, X.-J. Ma and Y. Luo, *J. Mol. Diagn.*, 2012, **14**, 22-29.
79. C. Wu, M. Simonetti, C. Rossell, M. Mignardi, R. Mirzazadeh, L. Annaratone, C. Marchiò, A. Sapino, M. Bienko, N. Crosetto and M. Nilsson, *Commun. Biol.*, 2018, **1**, 209.
80. C. Larsson, J. Koch, A. Nygren, G. Janssen, A. K. Raap, U. Landegren and M. Nilsson, *Nat. Methods*, 2004, **1**, 227-232.
81. F. Chen, A. T. Wassie, A. J. Cote, A. Sinha, S. Alon, S. Asano, E. R. Daugharthy, J.-B. Chang, A. Marblestone, G. M. Church, A. Raj and E. S. Boyden, *Nat. Methods*, 2016, **13**, 679-684.
82. G. Wang, J. R. Moffitt and X. Zhuang, *Sci. Rep.*, 2018, **8**, 4847.
83. G. Sun, J. Wan and H. Lu, *Biomicrofluidics*, 2019, **13**, 064101.
84. R. Ardeshiri, L. Hosseini, N. Amini and P. Rezai, *RSC Adv.*, 2016, **6**, 65714-65724.
85. K. Chung, Y. Kim, J. S. Kanodia, E. Gong, S. Y. Shvartsman and H. Lu, *Nat. Methods*, 2011, **8**, 171-176.
86. A. Zabihihesari, A. J. Hilliker and P. Rezai, *Integr. Biol.*, 2020, **11**, 425-443.
87. A. Nady, A. R. Peimani, G. Zoidl and P. Rezai, *Lab Chip*, 2017, **17**, 4048-4058.
88. F. Yang, C. Gao, P. Wang, G.-J. Zhang and Z. Chen, *Lab Chip*, 2016, **16**, 1106-1125.
89. H. B. Atakan, F. Ayhan and M. A. M. Gijs, *Lab Chip*, 2020, **20**, 155-167.
90. L. Dong, M. Cornaglia, T. Lehnert and M. A. M. Gijs, *Lab Chip*, 2016, **16**, 574-585.
91. Y. Cho, D. A. Porto, H. Hwang, L. J. Grundy, W. R. Schafer and H. Lu, *Lab Chip*, 2017, **17**, 2609-2618.
92. C. E. Stanley, J. Shrivastava, R. Brugman, E. Heinzelmann, V. Frajs, A. Bühler, D. van Swaay and G. Grossmann, *Bio-protocol*, 2018, **8**, e3010.
93. K. N. Badhiwala, D. L. Gonzales, D. G. Vercosa, B. W. Avants and J. T. Robinson, *Lab Chip*, 2018, **18**, 2523-2539.
94. K. Chung, M. M. Crane and H. Lu, *Nat. Methods*, 2008, **5**, 637-643.
95. M. M. Crane, J. N. Stirman, C.-Y. Ou, P. T. Kurshan, J. M. Rehg, K. Shen and H. Lu, *Nat. Methods*, 2012, **9**, 977-980.
96. T. V. Chokshi, D. Bazopoulou and N. Chronis, *Lab Chip*, 2010, **10**, 2758-2763.
97. H. Ma, L. Jiang, W. Shi, J. Qin and B. Lin, *Biomicrofluidics*, 2009, **3**, 044114.
98. H. Lee, S. A. Kim, S. Coakley, P. Mugno, M. Hammarlund, M. A. Hilliard and H. Lu, *Lab Chip*, 2014, **14**, 4513-4522.
99. J. Wan, G. Sun, J. Dicent, D. S. Patel and H. Lu, *Lab Chip*, 2020, **20**, 266-273.
100. S. M. Shaffer, R. P. Joshi, B. S. Chambers, D. Sterken, A. G. Biaesch, D. J. Gabrieli, Y. Li, K. A. Feemster, S. E. Hensley, D. Issadore and A. Raj, *Lab Chip*, 2015, **15**, 3170-3182.
101. M. A. Churgin, S.-K. Jung, C.-C. Yu, X. Chen, D. M. Raizen and C. Fang-Yen, *eLife*, 2017, **6**, e26652.
102. J. N. Pitt, N. L. Strait, E. M. Vayndorf, B. W. Blue, C. H. Tran, B. E. M. Davis, K. Huang, B. J. Johnson, K. M. Lim, S. Liu, A. Nikjoo, A. Vaid, J. Z. Wu and M. Kaeberlein, *GeroScience*, 2019, **41**, 961-973.
103. W. E. Pittman, D. B. Sinha, W. B. Zhang, H. E. Kinser and Z. Pincus, *Lab Chip*, 2017, **17**, 3909-3920.
104. J. C. K. Leung, A. J. Hilliker and P. Rezai, *Lab Chip*, 2016, **16**, 709-719.
105. D. Kim, M. Alvarez, L. M. Lechuga and M. Louis, *eLife*, 2017, **6**, e27057.
106. J. Larsch, D. Ventimiglia, C. I. Bargmann and D. R. Albrecht, *PNAS*, 2013, **110**, E4266-E4273.
107. A. Z. Shorr, U. M. Sönmez, J. S. Minden and P. R. LeDuc, *Lab Chip*, 2019, **19**, 1141-1152.
108. A. A. Popova, D. Marcato, R. Peravali, I. Wehl, U. Schepers and P. A. Levkin, *Adv. Funct. Mater.*, 2018, **28**, 1703486.



How can microfluidics address the significant limitations to the current tools that measure gene expression for single-animal studies?