Tick-tock, it's RNA o'clock

There's an evolving choice of ways to track the temporal dynamic of RNA biographies.

Vivien Marx

Without its dynamic to-and-fro, life would be boring. Researchers don't seem bored when assessing how cells dynamically change during cell division or differentiation. RNAs are important influencers in this dynamic. Each RNA has a life cycle that is quite a journey, says Harvard Medical School researcher Ruslan Soldatov. An RNA is synthesized, processed, transported from where it is made to other places in a cell, translated into proteins and then degraded. Cellular machineries actively regulate each individual step.

A cell's transcriptomic state sits at the center of genetic flow from DNA to protein, says Ling-Ling Chen, whose lab is at the Chinese Academy of Sciences Center for Excellence in Molecular Cell Science. Fixed samples show many examples of 'non-random' RNA localization patterns, and it begs the question "how did the RNA get there?" says Georgetown University researcher Esther Braselmann. Localization at a particular moment in time is likely part of what helps the RNA in its task. In each human cell, with its hundreds of thousands of mRNA molecules that maintain cellular and organismal function, the clock ticks faster for RNAs than for proteins; RNAs have a shorter molecular half-life, says Rockefeller University researcher Junyue Cao. For most genes, RNA lifetime is around three to four hours; it ranges from half an hour to over 100 hours. Different stages of the RNA lifespan also vary in terms of length and across genes, cell types, states and RNA species, to name a few aspects. "It's definitely mysterious why mRNA turns over so rapidly," says Samie Jaffrey, a researcher at Weill Cornell Medical College. It's constantly synthesized and degraded, usually over just a few hours, while proteins can last for days. "This type of behavior suggests that the cell wants to be able to rapidly increase or decrease RNA levels and doesn't want old mRNA sticking around."

Single-cell RNA-sequencing can do plenty, but it can't tell RNA time and discern whether an RNA is a veteran or a newborn. The method, says Cao, captures gene expression levels, but is limited in how well it reveals dynamics such as synthesis and degradation rates. Those factors are needed to better understand how mRNA molecular homeostasis is maintained and how disease sets it off-kilter.

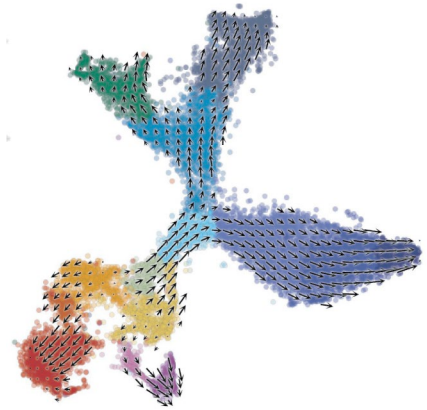
The clock ticks faster for RNAs than for proteins. How fast? scRNA-seq alone can't tell RNA time, but other emerging ways can. Credit: B. Curie Photography / Getty

Given the "desire to understand dynamic processes in tissues," labs have developed and used different ways to tell RNA time, says Sten Linnarsson from the Karolinska Institute Science for Life Laboratory. Among the RNA time-telling methods are computational ones that work with the "first time derivative of the gene expression state," as the developers of Velocyto, a computational approach nicknamed RNA velocity, phrase it; other approaches apply metabolic labeling followed by sequencing, and there are imaging-based methods. Beyond these, says Linnarsson, are methods for "understanding the life history of cells" such as MAESTER, which leverages endogenous somatic mutations, or "time-travel experiments" with Rewind, which combines RNA fluorescence in situ hybridization and barcoding.

Beyond snapshots

"Even in homeostasis, most tissues constantly turn over, and to understand the relationships and cell-state trajectories, you need methods that capture change, not just state," says Linnarsson.

Virtually all biological processes require cells to correctly tune the amounts of RNAs for a bunch of genes, which is usually achieved through regulation of some steps of the RNA life cycle, says Soldatov. Quantitative knowledge of the RNA life cycle would touch on many biological systems. For example, it yields information about how RNA clocks are adapted to enable quick response to an external stimulus, such as during development and regeneration, or during evolutionary time. "Analogously, how RNA clocks are dysregulated in diseased tissues and during aging are far from being well described," he says. Fortunately, experimental technologies and accompanying computational ideas emerging in recent years bring high hopes to digitize the cycle of RNA life. RNA velocity[1], he says, is based on the physics concept of velocity, which is the rate of change in an object's position over time. Here it's about capturing the rate of change in a cell's RNA amount over time. The concept



Velocyto is a computational way to measure RNA velocity, the rate at which RNA changes in a cell. The team used their method to plot future cell fates. Here is their t-SNE plot of developing mouse hippocampal cells. Adapted with permission from ref. [1], Springer Nature.

of RNA velocity underlies the widely used computational tool Velocyto for estimating transcriptional dynamics using a single-cell RNA-seq dataset. The result indicates "the immediate future state of the cell," says Linnarsson. The developers, including his lab, the Kharchenko lab at Harvard Medical School, Soldatov and others, used it to show changes in the developing mouse hippocampus. And Linnarsson says that Fabian Theis and his team at Helmholtz Center Munich are generalizing the use of RNA velocity for characterizing transient cell states with the computational approach scVelo.

RNA velocity has shown, says Soldatov, that single-cell measurements yield data about a cell's RNAs, which are all at slightly different stages of the RNA life cycle, and how one can use such data to predict cell dynamics. "In that aspect, it was an interesting reversion: traditionally scientists relied on known cell trajectories to explore RNA turnover, whereas RNA velocity models RNA turnover to explore unknown cell trajectories," he says.

In the wake of RNA velocity, methods have emerged that use sequencing to obtain readouts. With RNA timestamps[2], RNA is tagged with a 'recorder motif' that chronicles accumulated adenosine-to-inosine edits made by RNA-editing protein. The changes are read out with sequencing.

Labeling techniques provide measurements of RNAs at a few stages of the RNA life cycle or at a handful of time points, says Soldatov. The RNA timestamp technique measures age of individual RNA molecules and presents "a full history of RNA events." The method is, he says, not yet scaled to measure all genes as other labeling approaches can.

The success of single-cell RNA sequencing (scRNA-seq) might have accelerated development of labeling techniques. "With scRNA-seq we suddenly started seeing snapshot of cell states of any biological system in great details," he says. But scRNA-seq destroys a cell to measure its RNA content only once, "so we couldn't know how the cell behaves in time." Consequently, the snapshot of cells it delivers is essentially static. At the same time, understanding cell trajectories in dynamic biological systems has "quickly become of paramount interest in the community," he says.

Instead of this snapshot, capturing mRNA dynamic rates more faithfully reflects the cellular response to internal and external perturbations, says Cao. For example, identifying the response to short-pulse drug treatment involves newly synthesized mRNA, given that the global transcriptome shows almost no change. Cells at two distinct cell cycle phases, such as early G1 and late G2/M phase, have nearly identical transcriptomes and only the synthesis rate of cell-cycle-specific genes hints at how they differ from one another.

Cao led the development of sci-fate[3] as a postdoctoral fellow in Jay Shendure's lab at the University of Washington. In sci-fate, newly synthesized mRNA is labeled with 4-thiouridine (4sU) followed by single-cell combinatorial indexing and sequencing (sci-RNA-seq)[4]. There are two steps to the method's combinatorial barcoding scheme, which tags single cells and single-nucleus transcriptomes. When the modified 4sU is incorporated, it becomes a template for guanine instead of adenine. The thymine-to-cytosine conversion that took place is trackable through sequencing.

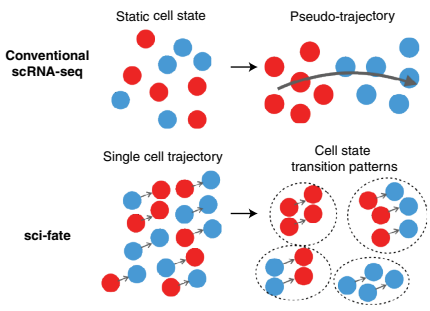
What Cao likes most about sci-fate analysis is how it lets researchers directly reconstruct "a beautiful single-cell transition circle across the main cell cycle phases from G1 to G2/M and back to G1 phase without any prior knowledge about the cell cycle." It also opens up the ability to quantify the transition rate between two cell states and lets researchers predict the cell population after a chosen amount of time. This technique and analysis could be a first step in "constructing a quantitative cell state transition network" that enables better modeling, which is needed to improve understanding of cell population dynamics in mammalian development and aging.

RNA that is newly synthesized and labeled, says Cao, can shed light on gene regulation mechanisms. For example, the data can directly be used to identify hundreds of regulatory links between transcription factors and genes. The approach captures the "velocity of transcriptome dynamics, from which we can infer the past and future state of each cell," he says. Researchers can use these data to study cell state transition probabilities across heterogenous cell states to characterize cell population dynamics in a complex system.

Sci-fate could evolve in a number of directions, he says. For example the team might integrate sci-fate with the method he co-developed called sci-RNA-seq3 to use single-cell combinatorial-indexing RNA sequencing, to profile millions of cells and to characterize cell state dynamics in a much more complex system, he says. Alternatively, sci-fate could be coupled with sci-CAR, a method he and colleagues developed to analyze RNA transcripts and single-cell chromatin profiles jointly, and capture transcriptome dynamics and epigenetic state in the same cell. That would be a way to study the effect of *cis*-regulatory elements, says Cao.

RNA velocity and sci-fate capture analogous information, and RNA velocity can be more straightforward to use, says Cao. The metabolic labeling needed in sci-fate might not always be possible but he and his colleagues believe sci-fate is more precise in terms of the timing aspects it captures. Velocyto can, says Soldatov, be run easily for any single-cell RNA-seq dataset, but it's "noisy and limited." Nucleoside labeling is an experimental technique that is "significantly more time-consuming and up to now limited to in vitro systems," he says, but it undoubtedly provides a "clearer portrait of the RNA life."

Cao and colleagues compared sci-fate to other RNA biography methods, notably scSLAM-seq and NASC-seq, and believe



In sci-fate, newly synthesized mRNA is labeled with 4sU, which leads to a thymine-to-cytosine conversion. Data are read out by single-cell combinatorial indexing and sequencing (sci-RNA-seq). Adapted with permission from ref. [3] Springer Nature.

sci-fate is cheaper to use and that it can measure more cells.

Separately, the developers of scNT-Seq[5] compared their method with others, including sci-fate. They note their method's similarity to sci-fate and its similar performance.

"SLAM-seq had such a strong influence on the development of techniques for measuring RNA lifetimes," says Jaffrey. "This is because SLAM-seq was so simple." In this method, scientists add 4-thiouridine to cells and then use the standard RNA-seq pipeline; there's no complicated library preparation. "It brought RNA stability analysis to the masses," he says.

Emerging labeling techniques, many of which are at single-cell resolution, dramatically expand capabilities to measure diverse stages of the RNA life cycle, says Soldatov. In his view, scEU-seq is an example of how biological insights about RNA life could be gained from this family of techniques. These methods are complementary to approaches such as RNA velocity or scVelo in that they aim to computationally model RNA turnover on the basis of measured stages of the RNA life cycle. Many of the ideas on which labeling techniques are based "were definitely devised independently of RNA velocity, so I wouldn't say that our method is the parent, but I hope it provides a sort of inspiring case," he says.

Getting labels to work

To assure metabolic labeling captures new RNAs, it "highly depends on labeling time and concentration," says Cao. He recommends labs test to optimize labeling efficiency to avoid damaging cells.

Given that metabolic labels are integrated into the RNA sequence when the RNA is transcribed, non-canonical nucleoside labels such as 4sU and 5-ethynyluridine can mark RNAs at birth, note Xing Chen, a researcher at Peking University in Beijing, and colleagues[6]. The labels can be conjugated to fluorophores for imaging or linked to affinity tags for enrichment and sequencing. But non-canonical nucleosides that work in eukaryotic cells often do not work in prokaryotes, which also have a dynamic transcriptome. For example, hundreds of noncoding RNAs regulate bacterial mRNAs and bacteria have riboswitches that let them quickly react to the world around them.

Xing Chen and his team have developed AIR-seq[6], a method in which a label called AzG is integrated into new RNAs and read out with sequencing on a genome-wide scale. The team used it to assess transcriptional dynamics in *Escherichia coli* in response to heat stress and to analyze changes to RNAs in the gut microbiota of live mice. They tracked changes that took place over minutes. In these experiments, *E. coli* only integrated one of the AzGs they developed, whereas *B. subtilis* was able to metabolize two of these label types. Perhaps it's a different tolerance to nucleoside kinases that shapes preference of bacterial species towards certain non-canonical nucleosides. "Before we have enough data or a complete understanding of the mechanism, it is recommended that one should evaluate the labeling when a new bacterial species is investigated," says Chen. And when labs use non-canonical nucleoside labels, whether in eukaryotes or prokaryotes, they should test to assure RNA function and dynamics stay unaffected, he says.

The team is now working on applying AzG to detect nascent bacterial RNAs during infection of a host with a pathogen. And they are developing a way to do large-scale identification of bacterial RNA-binding proteins.

In light of the range of RNA biography assessment methods, such as RNA velocity, sci-fate and scNT-seq, Chen sees exciting prospects for developing such methods for scRNA-seq in bacteria. Methods such as sci-fate and scNT-seq rely on the alkylation of 4sU to generate T-to-C mutations that are detected in nascent RNAs by scRNA-seq. AzG does not induce mutations. "Moreover, we suspect that chemical derivatization on the azide of AzG would not induce mutations either, because modifications on ribose probably do not affect base pairing," he says. But in his view it's desirable to develop non-canonical nucleosides to label bacterial RNAs that can modify nucleoside bases to generate mutations.

Imaging approaches

In live cells, RNAs have complicated behaviors just as proteins do, says Yi Yang, a researcher at the Shanghai-based East China University of Science and Technology. The lack of naturally occurring fluorescent RNAs that can be harnessed to study the dynamic molecular biology of RNAs is a challenge. But labs have worked out ways to do so following in the fluorescent protein tradition advanced by the late Roger Tsien and his team. They have developed approaches to fluorescent RNA labeling by selecting and engineering pairs of RNA aptamers and fluorogenic ligands, which fluoresce when the complex is formed. Ideally, says Chen, these genetically encoded fluorescent RNA aptamers should label and visualize diverse species of RNAs. "However, most previously reported aptamers have significant drawbacks," he says. Among the issues are weak fluorescence, instability, high non-specific fluorescence background, or the use of a ligand that cannot readily pass through the cell membrane and thus they cannot label function in live cells under normal conditions unless using, for example, beads or injection.

When studying RNA biographies, researchers should ask themselves about timescales and how perturbations might change expected dynamics, says Esther Braselmann.

Credit: G. Asakawa/ Univ. of Colorado

RNAs engineered to include motifs can be tethered to fusions of fluorescent proteins and specific RNA-binding proteins. But that can be a rather "heavy load" and there are issues with background fluorescence, the team points out[7]. The MS2 system, which involves a protein from MS2 bacteriophage and RNA-binding sequences, is the "gold standard" for RNA imaging in live cells and helpful for single-molecule studies.

Along with colleagues, Braselmann developed Riboglow[8] to tag RNA with a genetically encoded fluorescent probe in live single mammalian cells. The system leverages the bacterial cobalamin riboswitch as the RNA tag. When bound to RNA, a conformational change in the cobalamin–fluorophore probe occurs, and with it, fluorescence changes. The scientists engineered Riboglow to be smaller than the MS2 RNA tagging system, and thus more suitable for tagging smaller non-coding RNAs, she says. The team compared MS2, a method called Broccoli and Riboglow and found their tool easier to use than others. Riboglow has advantages, she says, but it cannot replace MS2. Overall, published tagging approaches are often specialized, and "transferring it to a different system might be difficult," she says. "I'm sure the same is true for Riboglow." For other researchers, it's "not always easy to assess how versatile a new method is beyond the advertised model system."

When studying RNA biographies, she says, researchers should draw on previous studies to put their experiments into context. They will want sufficient information about the tagged RNA to "confidently confirm" with control experiments that the tag will not affect an RNA's typical function and localization. "We also need to have a general sense of the types of RNA dynamics we

expect to detect when tracking tagged RNA live," says Braselmann. Scientists should know the localization patterns they are looking for and ask themselves, "What are the timescales? What perturbations might change the expected dynamics?"

As Jaffrey and his team note[9], few fluorogenic RNA aptamers have been developed because of the dearth of dyes for imaging RNAs in live cells. Dyes can light up non-specifically owing to interaction with lipids or DNA, and they tend to need to be added exogenously.

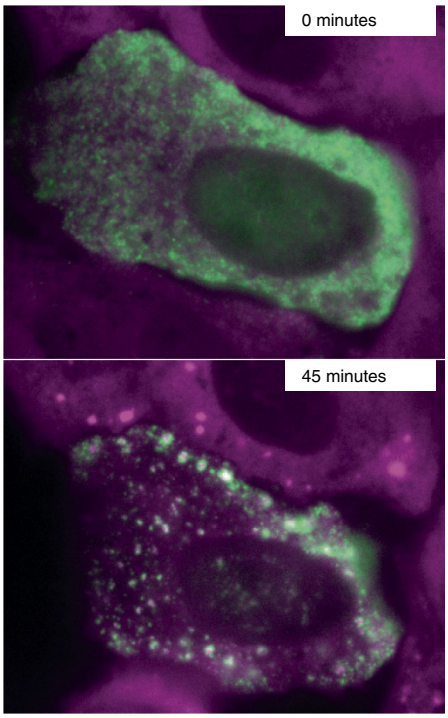
He and his group developed a tagging approach called Pepper, a set of fluorogenic proteins activated by RNA aptamers. The system uses a fluorescent protein, an RNA-binding peptide and a degron sequence, tDeg, which is a domain that promotes protein degradation. Fluorescence occurs when the fluorescent protein is bound by the RNA aptamer. This binding inhibits protein degradation, but when Pepper is unbound from the RNA aptamer, the protein is rapidly degraded in cells. Pepper is part of the lab's vegetable-named family of sensors that includes Spinach and Broccoli, among others.

The team found that the tagged mRNA behaved as endogenous RNA would and the Pepper tag "did not substantially" affect the stability, translation or localization of the mRNAs the scientists tested. The team notes that the tags are best in experiments that compare mRNAs that differ by a single sequence alteration or that assess the same mRNA in two different conditions.

It's "elegant design," says Yang of the system. Jaffrey's Pepper–tDeg system has multiple components, and its advantage is that it's fully genetically encoded and can use fluorescent proteins from blue to infrared as reporters, he says.

The Yang lab along with colleague Linyong Zhu and team, also at East China University of Science and Technology, developed an RNA tagging system that they also call Pepper. It uses synthetic dyes they developed. The Yang–Zhu team's Peppers are fluorescent RNA probes that have been commercialized and are currently available from cyan to red. In the team's view, their Pepper is faster to fluoresce, is more sensitive and has less background noise than Jaffrey's Pepper–tDeg system. But in the Yang–Zhu system, cells need to be cultured in or incubated in medium containing the synthetic dye.

As Jaffrey explains, the two Peppers are completely dissimilar. The Pepper from his lab, he says, can bind and stabilize a fluorescent protein that otherwise would degrade rapidly. "Our Pepper approach allows fluorogenic aptamers to move away from small-molecule dyes and move towards genetically encoded fluorescent proteins," he says. His lab's system enables full genetic encoding, which can be important for imaging RNA in a living animal without the potential toxicity of the small-molecule dyes. "The Pepper fluorogenic aptamers from the Yang lab are very elegant and represent a continued optimization of the fluorogenic aptamer technology," says Jaffrey.



The Jaffrey lab developed Pepper, a set of fluorogenic proteins activated by RNA aptamers. Here, Pepper is used in live cells to track β-actin mRNA condensing into stress granules over time. Adapted with permission from ref. [9], Springer Nature.

In Yang's view, although fluorescent RNAs have much improved brightness and signal-to-noise ratios compared to other live-cell RNA labeling technologies, it remains challenging to image RNAs in live cells. One hurdle is that RNA abundance is usually three to four orders of magnitude below that of proteins. This means that tracking RNA biographies in imaging experiments should be done in careful, well-controlled ways, says Yang.

Tracking circRNA time

Among the methods to track specific RNAs are ones that use RNA-binding protein systems with probes, says Liang-Zhong Yang, a graduate student in the lab of Ling-Ling Chen. He initiated a CRISPR–dCas13 system for RNA labeling in her lab[10].

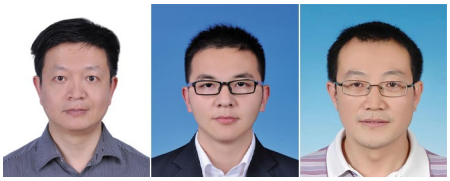
Tandem repeats of aptamers can be inserted into target RNAs and RNAs can be visualized by expressing fluorescently labeled aptamers that bind proteins or dyes, but concerns remain—for example, whether this inserted sequence might influence the RNA's functions, says Yang. Programmable RNA tracking systems such as ones using CRISPR–dCas13 and RCas9 have shown their "huge convenience in tracking whatever RNAs we want." These systems can handle both overexpressed exogenous and unmodified endogenous RNAs.

But before scale-up for use in live-cell RNA tracking, says Yang, there's high background to contend with. Researchers should, he says, gauge and test the influence of their system: the dynamics of the targeted RNA might be affected, translation might be altered and binding with RNA-binding proteins might change behavior. Ling-Ling Chen agrees. She is particularly interested in circular RNAs (circRNAs), and tracking these RNAs is especially tricky. She developed a way to capture the non-polyadenylated RNAs and, later, an assay for screening circRNAs using the CRISPR–RfxCas13d system.

Chen and her group have explored ways to visualize circRNAs in living cells. What makes this tough is that circRNAs from backspliced exons share the exact same sequences as their cognate linear RNAs, except for the backsplice junction site. Another challenge: circRNAs are expressed at lower levels than linear ones, she says.

Theoretically, she says, one could splice an aptamer on each side of the backsplice junction site into a circRNA expression vector, or target the backsplice junction site directly with programmable RNA tracking systems. Thus far, existing labeling systems show insufficient efficiency and resolution, she says, and given the low abundance of many circRNAs, researchers should apply methods to track circRNAs with caution and optimize specifically for circRNAs. She and her team are working to synthesize round elements that carry different aptamers. "Hopefully, she says, by combining such approaches with synthetic biology "we will be able to 'see" circular RNAs in cells in a not-so-distant future."

Given that metabolic labeling can change RNAs, says Yang, researchers are advised to test, try and compare multiple methods to track RNAs. Chemical modifications may indeed introduce unwanted mutations that might affect local conformation of RNAs, says Chen. Inserting aptamers into individual RNA loci might even dampen or enhance local RNA transcription, thereby introducing artifacts into the system. Given the different pros and cons of methods, people can consider applying multiple



At East China University of Science and Technology, Yi Yang, Xianjun Chen, Linyong Zhu (from left to right) and colleagues have developed fluorescent RNA probes and dyes called HBCs.

approaches to the same target to reach a general conclusion, she says.

Just as RNA can be used to distinguish unspliced from spliced mRNAs, it might be possible to develop circ-velocity to distinguish among circRNAs, says Yang. Unlike RNA velocity, methods such as sci-fate and scNT-seq need 4sU labeling to detect nascent mRNAs. But, he says, in some situations, longer treatment is needed and 4sU ends up labeling most RNAs, not only nascent ones.

Next moves

Ever since the use of fluorescent proteins began, the tools' lineage has evolved, with expanded color space, enhanced brightness and added features such as photoconvertibility or photoswitchability, says Yi Yang. Beyond their use as tags, these fluorescent proteins are used in genetically encoded sensors of many kinds, as well as in optogenetic tools for controlling protein function. "Our story of fluorescent RNAs is just beginning," says Yang.

Fluorescent RNAs make it possible to image low-abundance RNAs in live cells, but it's still challenging to render RNAs visible in a multiplex way or in vivo. Yang believes fluorescent RNAs will evolve much as fluorescent proteins have, with more colors, such as infrared, and they will be engineered to be brighter, photoconvertible or photoswitchable. They could be useful for multiplexed, sensitive, single-molecule or super-resolution microscopy–based detection of RNAs in live cells and in vivo. Technologies that use fluorescent RNAs to make genetically encoded sensors or optogenetic actuators are also emerging, he says, and compared to fluorescent proteins, fluorescent RNAs have a much smaller coding sequence and simpler structure, which opens up many potential applications.

Both in terms of process and purpose, the methods that involve sequencing are quite unlike approaches such as the fluorescent RNA from his lab, says Yang. Methods such as sci-fate, scSLAM-seq, scNT-seq, TimeLapse-seq or TUC-seq and others that involve metabolic labeling and sequencing are useful for transcriptome-scale RNA profiling, says Yang. He has heard that biology labs can find it tricky to perform nucleoside labeling and that spatiotemporal information is difficult to acquire with those methods. But there's "no doubt" that sequencing-based techniques provide transcriptome-wide information about RNA generation or degradation.

Fluorescent-RNA-based labels and imaging help investigators assess spatiotemporal dynamics of single RNA species in live cells. "I would say these methods are complementary but not competing" says Yang. If a researcher is doing a survey-type 'omics study, the sequencing-based methods can be used, and then fluorescent-RNA-based approaches can offer a deeper understanding of a few specific RNAs. Researchers might use sequencing methods first and fluorescent RNAs later or vice versa, he says.

His focus is more on computational aspects, says Soldatov, but it seems to him that combining different metabolic labels promises a way to expand the time range of measurements. The labeling techniques are, to his knowledge, mainly limited to in vitro experiments, and adapting them to in vivo systems, he says, "would be an important cornerstone."

"Plenty will yet emerge in the RNA time-analysis space," says Linnarsson. Next up is monitoring rich cell-state trajectories in live tissues such as organoids or explants, or, for in vivo settings, "essentially getting to the equivalent of live single-cell RNA-seq but by microscopy." That gives scientists a direct gaze on "true differentiation trajectories, linking stem cells to differentiated cell states over long periods of time and long spatial distances," he says. These data will be three-dimensional in the way they, for example, show cell–cell interactions and the whole-tissue context, such as niches.

Braselmann offers a hypothetical scenario for combining methods. Metabolic labeling of an RNA of interest can represent an unbiased approach to learning about RNA by minimally perturbing the RNA in question. And then researchers can follow up on this experiment by fluorescence in situ hybridization to home in on the RNA with minimal perturbation of the RNA sequence. Then, using a genetically encoded aptamer such as Riboglow for fluorescence-based single-molecule RNA tagging, they might then do a follow-up study to dissect more detailed live dynamics and test hypotheses about RNA dynamics in healthy cells and in perturbation settings. "These different techniques ask different questions and are all important in combination," she says. ❐

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