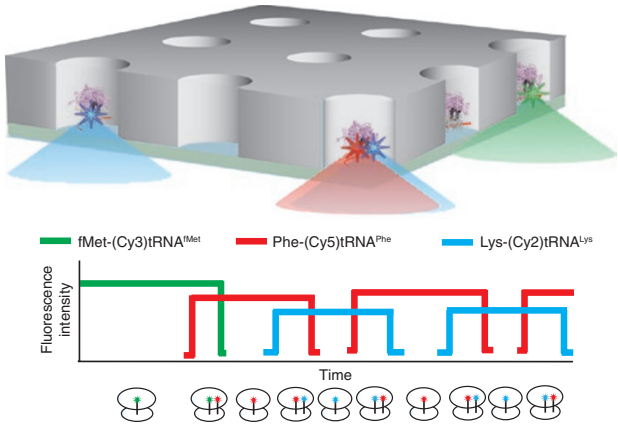
BIOPHYSICS

A glow on protein synthesis

Applying technology developed for next-generation DNA sequencing to study translation, researchers watch individual ribosomes string together amino acids in real time.

Since the 1950s, numerous studies have provided important insights into the process of translation, and achievement in the field was recognized with the 2009 Nobel Prize. Translation has even been dissected in single-molecule studies, albeit at lower than physiological concentrations. So what has been lacking is the ability to study the timing of translation at micromolar concentrations of components, on par with the conditions in the cell that enable this fast and efficient process.



As each labeled tRNA binds the ribosome immobilized in a ZMW, a clearly separated signal is recorded. Occupancy of ribosome sites by tRNAs is extrapolated by monitoring fluorescence signals over time. Image courtesy of Joseph Puglisi and Sotaro Uemura.

To begin to address what Joseph Puglisi of the Stanford University School of Medicine calls "the choreography of translation," his group collaborated with researchers at Pacific Biosciences—who perfected a technology for single-molecule, real-time studies in tiny (10^{-21} liter) reaction chambers. This is the technology used in the company's next-generation DNA sequencing process. Briefly, the enzyme is immobilized in chambers, called zero-mode waveguides (ZMWs). Enzyme activity is measured in real time as dye-labeled ligands bind the enzyme, within the detection volume. Of importance, background signal in this system is greatly reduced, making possible experiments with micromolar concentrations of ligands.

In recent work, the researchers immobilized in the chambers *Escherichia coli* ribosomes in the form of 70S initiation complexes containing a fluorophore-labeled initiating *N*-formylmethionine (fMet) tRNA. They also added tRNAs—two, in this proof-of-concept study—each labeled with a different dye. The tRNAs were cognate for the amino acids encoded in the test mRNA, phenylalanine and lysine.

At the start of the experiment, only the fMet tRNA signal is detected. As each subsequent labeled tRNA binds the ribosome, as dictated by the test mRNA, the fluorescence signal in the detection volume reports the

GENOMICS

BOUND TO THE MESSENGER

A new technique for transcriptome-wide isolation of RNAs bound to specific proteins reveals, with high resolution, the location of RNA-binding proteins on their target RNAs.

RNA transcripts are never alone in the cell. In particular, RNA-binding proteins (RBPs) and microRNA-containing ribonucleoprotein complexes bind mRNAs in a sequence-dependent manner and have an important role in regulating gene expression post-transcriptionally. Hundreds of these RBPs and microRNA-containing ribonucleoprotein complexes modulate the maturation, transport, editing and translation of mRNAs in the cell, but techniques for precise identification of where these molecular complexes are bound to the mRNAs *in vivo* are still lacking.

UV-light irradiation is known to covalently cross-link RNA to RNA-bound protein complexes in living cells. In an approach called cross-linking and immunoprecipitation (CLIP), live cells are exposed to UV light to freeze the RNA-protein interactions and allow isolation of proteins of interest together with their associated RNAs by immunoprecipitation. High-throughput sequencing of the reverse-transcribed, cross-linked RNA results in a set of sequence reads representing RNA sequences that were bound to the particular RNA-binding complex in the cell.

But this approach has some limitations. Photo-cross-linking with 254-nm UV light, as is done in CLIP, is a relatively inefficient process that leads to the difficult problem of separating signal from background. Biochemical separation of cross-linked from non-cross-linked RNA is a demanding process and still requires the use of material from gene knockout organisms, computational models or filtering of expected sites or motifs to combat the inherent noise.

To solve these problems comes the next generation of high-throughput RNA cross-linking, photoactivatable ribonucleoside–enhanced CLIP (PAR-CLIP), developed by Thomas Tuschl's group at the Rockefeller University in collaboration with the group of Mihaela Zavolan at the Biozentrum in the University of Basel. PAR-CLIP introduces the use of photoactivatable nucleosides as efficient and nontoxic cross-linkers that are well incorporated into the RNA. In this method, cells are grown in the presence of 4-thiouridine (4SU) and 4SU-substituted RNA is cross-linked using 365-nm UV light, which is safer and more efficient than cross-linking unsubstituted RNA using 254-nm UV light.

The true advance of this technique arose from a fortuitous finding: the cross-linked nucleosides cause a specific base change during reverse transcription that leaves a permanent mark in the place where the protein complex originally stood on the RNA. As the reverse transcriptase misincorporates guanine (G) opposite the cross-linked 4SU base, scoring for thymidine (T) to cytidine (C)

timing of the event, in addition to the identity of the tRNA. Fast sampling events at the A site can be distinguished from tRNA binding. Signal overlap indicates that two tRNAs are bound to the enzyme.

"That is really the grail of translational analysis," says Puglisi, adding that with this method "not only do you get information about the dynamics of translation as a biophysical measurement, but you also get the underlying sequence of the mRNA, so you get sequence-dependent behavior of translation." With this setup, the researchers addressed a long-standing question in the field, finding that tRNA release from the A and E sites is uncoupled.

And this proof-of-concept study is just the beginning. The group has already expanded the system to four dyes and is moving on to determine how drugs affect translation, among other questions. The system could also be used to analyze processes from initiation to frame-shifting as well as ribosomes from various species, including the eukaryotic ribosome. Pointing out the range of questions that can be addressed, Puglisi notes that one does not have to work with purified components but instead can use extracts doped with reagents.

Another important conclusion from this work is how extensible the ZMW technology is to various biological systems. Translation is far more complicated than DNA sequencing, which bodes well for extending the technology to other systems. For many systems—as was the case for the work reported here—many labeled reagents are already available, and it is just a matter of asking the right questions. "I just hope this technology gets in the hands of people in different fields so that they can start experimenting with it," concludes Puglisi.

Irene Kaganman

RESEARCH PAPERS

Uemura, S. *et al.* Real-time tRNA transit on single translating ribosomes at codon resolution. *Nature* **464**, 1012–1017 (2010).

transitions in the sequenced cDNA opens the door to precisely mapping the binding sites for RNA-binding proteins, and it allows the separation of bona fide cross-linked RNA sequences from noise.

Tuschl and co-workers performed a herculean demonstration of the powers of PAR-CLIP with transcriptome-wide mapping of binding sites for 13 RBPs, of which seven proteins are involved in microRNA targeting. By focusing on sequences that bear the hallmark T to C transition, the authors defined binding motifs for RBPs such as insulin-like growth factor-2 and identified previously unknown targets for microRNAs.

These data sets verify the robustness of the technique by confirming known binding partners, and also provide a wealth of new RNA-interaction maps. "The struggle is now to prioritize all this data-mining," says Tuschl. "We want to know if there are genetic variations in these binding sites in humans and whether some of them contribute to genetic diseases that implicate loss of an RNA-binding protein, such as fragile X mental retardation or familial amyotrophic lateral sclerosis."

The future of PAR-CLIP is bright, promising new and imaginative possibilities for experimentation that will bring science a step closer to unraveling the mysteries of gene regulation.

Erika Pastrana

RESEARCH PAPERS

Hafner, M. *et al.* Transcriptome-wide identification of RNA-binding proteins and microRNA target sites by PAR-CLIP. *Cell* **141**, 129–141 (2010).

NEWS IN BRIEF

PROTEIN BIOCHEMISTRY

Super-resolution crystal structures

It remains challenging to obtain large, well-diffracting crystals of many proteins; a method to obtain atomic structures from low-resolution X-ray diffraction data would therefore be welcomed. Schröder *et al.* describe such an approach, using information from known homologous structures to help interpret the electron density maps of low-resolution data, while allowing global and local deformations. The method improved 19 existing low-resolution structures to a quality similar to that of high-resolution structures.

Schröder, G.F. *et al. Nature* **464**, 1218–1222 (2010).

NEUROSCIENCE

Microfluidics gets to the synapse

Neuronal connectivity in synapses can be studied using dissociated neurons in culture. Taylor *et al.* now refine these studies by designing a microfluidic device that enables precise manipulation and visualization of synapses. In their setup, two neuronal populations are grown on separate compartments that are connected by microgrooves into which axons and dendrites grow, forming functional synapses. A small perfusion channel runs perpendicular to the microgrooves, allowing high spatial and temporal control over synaptic microenvironments.

Taylor, A.M. *et al. Neuron* **66**, 57–68 (2010).

GENOMICS

Mapping CNVs with base-pair resolution

To understand the function of copy number variants (CNVs), the CNV breakpoints must be characterized with base-pair resolution. Recognizing that high-throughput, cost-effective methods for breakpoint sequencing have been lacking, Conrad *et al.* use an oligonucleotide array to capture targeted genomic regions and then sequence the captured regions via 454 pyrosequencing. They identified the sequence breakpoints for 324 CNVs in three unrelated individuals, leading to new insights into mutational mechanisms.

Conrad, D.F. *et al. Nat. Genet.* **42**, 385–391 (2010).

MOLECULAR LIBRARIES

Engineering mammalian enzymes

Phage display is a powerful technology used in directed evolution for selecting protein variants with desired properties, but it is not well suited for displaying mammalian proteins with extensive post-translational modifications. Granieri *et al.* use a combination of retroviral display and water-in-oil–based droplet microfluidics to screen complex mammalian enzymes under multiple turnover conditions, by following the conversion of a nonfluorescent substrate into a fluorescent product.

Granieri, L. *et al. Chem. Biol.* **17**, 229–235 (2010).

SYSTEMS BIOLOGY

Mouse development, cell by cell

In the preimplantation mammalian embryo, development is driven by fate decisions occurring in few or even single cells. Using manual separation of single cells and Fluidigm chips, Guo *et al.* carried out expression analysis of 48 candidate developmental regulatory genes in more than 500 single cells at multiple time points over the first four days of mouse development. This resource should permit insight into the regulatory networks underlying the earliest lineage decisions in the mouse embryo.

Guo, G. *et al. Dev. Cell* **18**, 675–685 (2010).