GENE EXPRESSION

Transcript constellations in a tissue's universe

The STARmap method advances RNA sequencing in intact tissues.

Just as a night of stargazing calls for a clear sky, locating transcripts in the brain requires a clear mind. Tissue clearing has always been important for hybridization approaches that probe RNA molecules in thick tissue. While developing the CLARITY technique to visualize structures deep in the brain and other organs, Karl Deisseroth imagined an eventual use for sequence mapping. At a talk on the day that the work was published in 2013, he was asked whether he would adapt the approach for in situ RNA sequencing. "I said we would be working on it," he recalls. "Five years later, thanks to the creative team led by Xiao Wang and Will Allen, here we are."

The new work, supervised by Deisseroth along with fellow Stanford researchers Felice-Alessio Bava and Garry Nolan, presents a method called spatially resolved transcript amplicon readout mapping, or STARmap. It packs a number of advanced technologies into a single protocol.

In the first step, two oligonucleotide probes—a circular padlock probe that requires ligation to detect the transcript, and a second probe that primes rolling-circle amplification of the ligated padlock—hybridize to adjacent spots on an RNA target in fixed tissue. Amplification generates a ball of DNA containing many copies of a transcript-specific barcode. By requiring that both probes bind, the researchers boosted specificity, and by avoiding reverse transcription, they greatly improved the efficiency of transcript capture. STARmap is as sensitive as single-cell RNA sequencing.

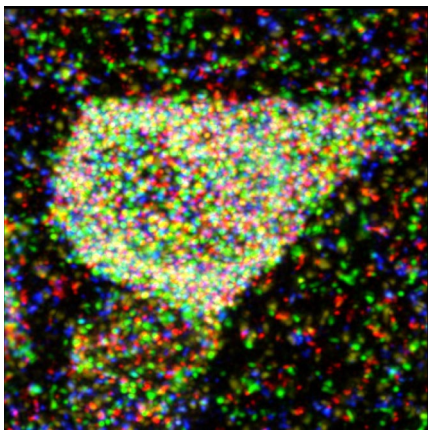
In the second step, tissue is covalently embedded in hydrogel. Similar to CLARITY, acrylamide monomers are infused into tissue, then polymerized and anchored to amplicons, which include functionalized nucleotides that bond with the polymer matrix. After the removal of lipid and protein, the amplicon-hydrogel hybrids are more stable and transparent. They have lower background fluorescence and allow rapid diffusion, and amplicons cannot drift during sequencing.

For the final step, the researchers developed a ligation-based sequencing strategy. To overcome inaccuracies and issues with background in existing approaches, they used a two-probe design to read out barcodes. A short 'reading probe' determines which nucleotides are available for stable hybridization and ligation of a 'decoding probe' that carries two complementary bases and one of four encoded fluorophore colors. In each cycle, bound probes are stripped and a new reading probe shifts the sequencing position by one base. The barcode is decoded as a color sequence, and an erroneous color causes the final base, which flanks the barcode, to be read incorrectly, signaling an error. The strategy reduces error rates dramatically, to below 2%, though at a cost of discarding 20–40% of imaged amplicons.

The STARmap protocol transforms a piece of tissue into a coded universe of bright, multicolored spots that can uncover relationships between anatomy and function. As an initial test, the researchers targeted a collection of cell-type- and neural-activity-regulated markers, comprising 160 genes in total, in thin slices of adult mouse visual neocortex. The experiments identified all 12 major cell types, as well as the known spatial distribution of cortical layer markers and interneurons, with reproducible expression across biological replicates. They had similar success in the medial prefrontal cortex, and expanding their measurements to include 1,020 genes allowed them to identify three new cell types.

A five-base STARmap barcode scheme can represent up to 1,024 unique transcripts, and this number can be pushed upward. Yet at high expression levels, light from neighboring amplicons from the same gene cannot be resolved, leading to underestimates of RNA copy number. "Even so, we've observed up to 1,000 copies per gene per cell, and up to 10,000 amplicons per cell," says Deisseroth, noting that this could be improved using super-resolution methods.

A key strength of STARmap is its applicability to thick tissues, which leverages a higher signal-to-noise ratio than single-molecule fluorescence in situ hybridization. The scientists mapped 28 genes across all six layers of visual cortex and the corpus callosum in mouse, and found new spatial relationships between cortical neurons. A number of adjustments, such as collecting data at whole-cell resolution, sped up these experiments. Library generation and imaging take about 2 to 9 days, depending on tissue thickness and the number of targeted genes.



A single neuron being decoded for the spatial expression of 1,000 genes. Credit: Xiao Wang and Karl Deisseroth.

Further improvements are already in the works, such as optimized fixation of archived tissue. One exciting direction is the integration of other data types. "We are working on registering STARmap with functional imaging at single-cell resolution, so that gene sets in active cells can be linked to behavior," says Deisseroth. He expects that STARmap will not be restricted to particular tissues.

STARmap is providing a clearer picture of cellular function in its anatomical context. "[Our] findings on cell clustering and layering with STARmap have indicated that cell distances and boundaries can be most accurately assessed in three dimensions rather than two," says Deisseroth. "And this quantitative observation has key basic and clinical implications." ❐

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Research papers

Wang, X. et al. Three-dimensional intact-tissue sequencing of single-cell transcriptional states. *Science* https://doi.org/10.1126/science.aat5691 (2018).