MICROSCOPY

Fast volumetric imaging in live samples

Swept, confocally aligned planar excitation imaging is a fast light-sheet microscopy technique that can be applied to live samples such as behaving animals.

Imaging neuronal activity or other dynamic processes in awake animals is challenging owing to their movements, which can lead to artifacts during imaging. To overcome this problem, Elizabeth Hillman and her collaborators at Columbia University developed a high-speed volumetric imaging technique inspired by advances in light-sheet microscopy.

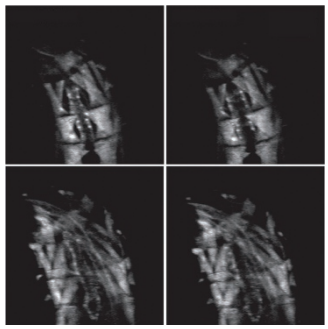

Image sequence of a crawling *Drosophila* larva with GFP-labeled muscles and central heart tube. Image reproduced from Bouchard *et al.*, Nature Publishing Group.

The idea of applying light-sheet microscopy to dynamic samples initially seemed crazy to Hillman because typical samples for light-sheet microscopy are dead, and thus there is generally no need for fast live imaging. The design of light-sheet microscopes requires the alignment of two or even four objectives, imposing sample constraints. Hillman and her colleagues came up with a microscope design that gets by with a single objective. The light sheet exits the objective from the edge and illuminates the sample at an oblique angle. The same objective collects the emitted light from the illuminated plane. With these modifications, "you don't need to prepare your sample, you don't need to hold your sample," says Hillman, making it possible to image live animals.

The increase in imaging speed necessary for live imaging required further adaptations compared to established fast light-sheet microscopy implementations. Traditional

BIOINFORMATICS

KINDRED CELLS AMONG THE CROWD

Modeling cell-cycle state from the transcriptional profiles of single cells can improve the ability to group cells by function.

Over coffee one day at the European Molecular Biology Laboratory–European Bioinformatics Institute in Cambridge, UK, where they work, John Marioni, Oliver Stegle and Sarah Teichmann discussed the issue of heterogeneity in populations of dividing cells. The conversation wandered to the cell cycle. For the RNA of a single cell to be sequenced, that cell must be sacrificed, and along with it, any chance of testing other cellular properties—how would it be possible to know the cell-cycle status of the cell or the effect it had on gene expression?

The goal of many single-cell sequencing studies is to group cells by gene expression profile into subpopulations with a common identity or function. But cells may also share transcriptional features because they come from the same experimental batch or are captured at the same point in the cell cycle, obscuring other biological signals.

To model the effect of these confounding factors on gene expression, Stegle and Marioni looked to the field of statistical genetics, which grapples with the effect of cryptic genetic relationships in sampled populations. "A lot of methods that are analogously used in human genetic studies were using relatedness effects with individuals," says Marioni.

The single-cell work began when Florian Buettner joined the groups on a short-term visitor fellowship. The data presented unique challenges. "Arguably the biggest problem with single-cell data is dealing with the technical noise," which is far higher than genotyping error in human studies, says Marioni. They used their prior method for noise detection to identify genes expressed above technical noise and with enough variability to discriminate between cell types. Of these, they selected a set of cell cycle–correlated genes.

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setups can reach a volumetric imaging speed of 1 Hertz by moving the imaging plane with the help of an electrically tunable lens or piezoelectric translation. Hillman and her colleagues achieved a higher imaging speed of 20 volumes per second by keeping the stage and the objective stationary. To acquire image volumes, they swept the light sheet through the sample by reflecting the light from a slowly rotating polygonal scanning mirror. Coincidentally, the light returning from the sample was similarly deflected, thereby maintaining the alignment of incoming and outgoing light paths. In light of these two modifications, the researchers named their technique swept, confocally aligned planar excitation (SCAPE) microscopy.

"The system we have now is so versatile and usable that you can literally just stick anything under there," says Hillman. Together with her collaborators, she demonstrated the capabilities of the SCAPE microscope by imaging neuronal activity in head-fixed, awake mice and in crawling *Drosophila* larvae. The high imaging speed allows monitoring of calcium activity in large volumes, which was not possible with imaging techniques that are traditionally used for calcium imaging in animals, such as laser scanning confocal imaging.

Hillman thinks that, in comparison to other fast imaging techniques, SCAPE microscopy has two main advantages. First, neither the objective nor the sample is moved, which makes the technique exceptionally fast but also does not disturb the sample in any way. And similarly to other light-sheet microscopy techniques, only a single plane is illuminated at any given time, thereby lowering phototoxicity, which is important in live imaging.

Although the current implementation of the SCAPE microscope works well for calcium imaging, the group is continuously improving the system, especially because it does not yet reach the resolution that is theoretically possible. "I am pretty confident that we can reduce the number of lenses in the system and actually have it work better," says Hillman.

Nina Vogt

RESEARCH PAPERS
Bouchard, M.B. *et al.* Swept confocally-aligned planar excitation (SCAPE) microscopy for high-speed volumetric imaging of behaving organisms. *Nat. Photonics* **9**, 113–119 (2015).

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The team built a single-cell latent-variable model that first reconstructs the cell-cycle state using this gene set and then uses the information to correct expression levels, which can be plugged into existing analysis tools. It also estimates the proportion of variation attributable to cell cycle and technical noise. The approach worked well on nuclear dye–labeled embryonic stem cells from mouse in which cell-cycle status was detected before sequencing.

Next, they examined data from a mixed population of rapidly dividing naïve and differentiating T helper cells. Using their approach, they clearly detected two subpopulations differentially enriched for genes involved in immune cell differentiation, whereas clustering after simply removing genes involved in cell cycling could not discriminate these cell types.

The method can be used to model any potentially hidden factor, either to remove confounding factors or to identify a biological effect of interest. It can also accommodate multiple factors; for example, the researchers modeled cell cycle in addition to a set of genes associated with T helper 2 cells to better understand the differentiation process. The tool may be limited when sequencing is very shallow, but they showed that detecting as few as ten cell-cycle genes gives robust performance.

As single-cell expression data become more common, the advantages of more sophisticated approaches to model transcriptional heterogeneity within cell populations are becoming apparent. "Obviously it's worked better than we might have anticipated when we had that coffee conversation," says Marioni.

Tal Nawy

RESEARCH PAPERS
Buettner, F. *et al.* Computational analysis of cell-to-cell heterogeneity in single-cell RNA-sequencing data reveals hidden subpopulations of cells. *Nat. Biotechnol.* **33**, 155–160 (2015).