Improved multi-label imaging using hyperspectral phaser analysis

Software for separating spectra helps scientists, and potentially clinicians, sort through the signals.

The need to identify friend and foe is nowhere more apparent than in the operating room, where surgeons work diligently to distinguish and remove tumors with minimal damage to surrounding muscle and nerve tissue. New software developed by Francesco Cutrale and colleagues in the lab of Scott Fraser at University of Southern California could potentially aid this process, and along the way, help scientists make the most of a variety of imaging tools in zebrafish and mice.

For Cutrale, translating his work in microscopy methods across scientific disciplines and into the clinic is what it's all about. "Biologists use genetics in animal models, because the genetics can be somewhat translated back to humans. In my case, I'm ignorant of genetics, but I work on a core, which is an algorithm, and that algorithm can be translated between fields, and that is the real strength of this method." The algorithm he developed and packaged into a new software platform, called HyperSpectral Phasors—or HySP for short—allows unambiguous unmixing of multiple fluorophores with overlapping spectra, and significantly improves time-lapse *in vivo* imaging of multiple cell and tissue types (*Nat. Methods* **14**, 149–152 (2017)).

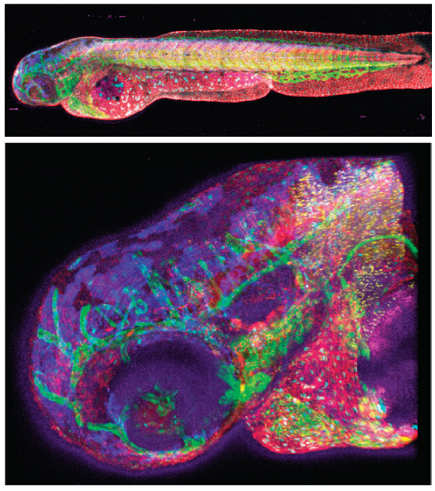
Simultaneous imaging of multiple structures and cells in living whole organisms has advanced greatly owing to continuous improvements in fluorescent probes. But several challenges still await the scientist that dares make use of more than two or three separate labels in the same tissue.

First, although researchers have a wide variety of probes to choose from, many have overlapping excitation and emission spectra, making it difficult to accurately distinguish one from the other in resulting images. Second is photobleaching and subsequent damage to living tissue, which increases with additional probes and excitation lines. Additionally, autofluorescence can be a significant factor affecting the quality of images, especially *in vivo* where a variety of structures with different cell types and densities can create difficult backgrounds.

Lastly, there is the issue of speed. Previous algorithms have been developed to de-noise data and solve some of the above problems, but are computationally intense and require far too much processing time, especially for live imaging experiments where close to real-time data is important. Working off of previously developed phasor analyses for multispectral imaging, which use computationally efficient fourier transformations of data, Cutrale *et al.* developed HySP and demonstrate its ability to quickly and accurately separate up to eight unmixed signals in developing zebrafish embryos over long imaging periods.

HySP takes the high-dimensional multispectral information for each pixel in an image, transforms it into a single point on a 2D phasor plot, then applies an algorithm that rapidly reduces spectral noise, removes autofluorescence, and cleanly separates multiple signals from fluorescent markers with overlapping spectra. As demonstrated in their paper, images with multiple labels are significantly improved. Importantly, the denoising process enabled images to be collected under low signal-to-noise conditions, allowing for low laser levels to be used during time-lapse live imaging, with no noticeable damage to growing zebrafish embryos.

For Cutrale and colleagues, this proof-of-principle is just the beginning. They see several future opportunities to apply their algorithm in a wide range of fields. The team is working on developing a semi-automated version of the algorithm that would enable scientists to more easily take advantage of tools like brainbow mice and zebrafish for lineage analysis. With some small tweaks to the software, they hope to add some user-friendly features that can identify and isolate cells with the same combination of colors, allowing researchers to simply switch channels and follow daughter cells with distinct origins.



Maximum intensity projections showing seven unmixed signals *in vivo* in a zebrafish embryo. Adapted from *Nat. Methods* **14**, 149–152 (2017).

Ultimately, Cutrale's game plan is to help push HySP into the surgery room, and he and colleagues in the Fraser Lab are already in discussion with industry leaders like Intuitive Surgical Inc., which developed the Da Vinci Surgical System, a robotic surgical platform now commonly used for prostate surgeries. As Cutrale notes, "They already have the interface for this; it's all digital, so the surgeon doesn't touch the patient anymore. He is sitting somewhere else looking at an image. What if we could augment that image to better separate, for example, muscle from nerve?"

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