If Only We Could Make Every Photon Talk

■ *By seeking to make every photon tell a tale, instrument makers are on the verge of providing genomics and proteomics researchers a tool to tease out more of nature's secrets. Hyperspectral imagers allow the use of multiple, overlapping fluorochromes and can remove sources of noise.* ■ **By Hank Hogan**

When her son played soccer, Maggie Werner-Washburne, PhD, a molecular biologist, University of New Mexico, Albuquerque, regularly watched the games. She could easily tell the teams apart because they wore different colored uniforms. But what if she had only been able to see a part of the spectrum, a fixed band? Then distinguishing between teams would impossible for some color combinations. Spectators who wandered onto the field might be hard to spot and getting an accurate count of the players might be difficult.

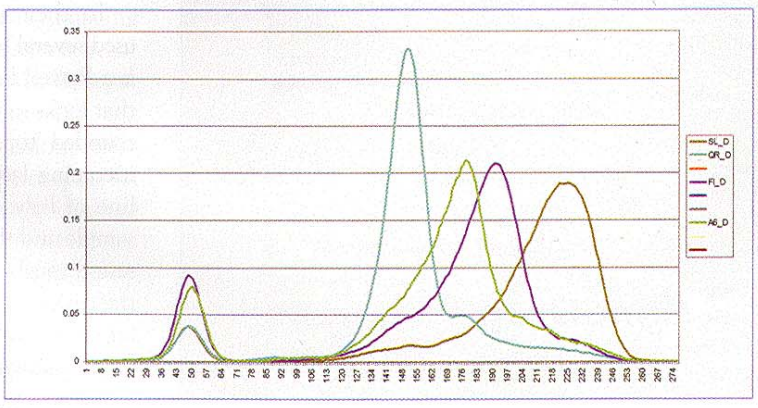
Yet, in some ways, commercial microarray scanners look at the world in just that fashion. Such scanners excite an array of spots with a light source and capture the resulting fluorescence. The emission is used to study gene expression.

Werner-Washburne, for example, researches the genes that control the exit from the stationary phase in yeast. This involves tagging genes and feeding starving cells. With a standard microarray scanner, identifying the changes can be challenging.

"Because we're looking at just a window of wavelengths, anything that is fluorescent in that wavelength would contribute to the signal," says Werner-Washburne.

In the late 1990s, at one of her son's soccer games Werner-Washburne started talking with a fellow team parent, David Haaland. A senior scientist, Sandia National Laboratories, Albuquerque, Haaland, PhD, specializes in infrared detectors.

After discussions about how they might work together, Haaland offered up the idea of using many different fluorochromes, a hyperspectral imager, and multivariate curve resolution. With a hyperspectral imager, the entire visible spectrum would be captured for every pixel. Multivariate curve analysis offered a way to mathematically untangle a combined spectral curve into its individual components, making it possible to use multiple and spectrally overlapping fluorochromes as well as to remove contributions from noise sources.



Spectrum data for four different fluorochromes, Quantum Red, Alexa 635, FITC (fluorescein), and a SL, a short-wavelength fluorochrome, captured with a superconducting tunnel junction (STJ) detector. The graph height (relative scale) shows the number of photons detected at each energy level (bottom axis, 0 energy at left). Peak around 50 (relative scale) is infrared, or heat, photon signal at around 1,100 nm wavelength from the fiber optic cable. The other peaks are fluorescent photons from the fluorochomes which have been separated, showing how the STJ detector is able to separate the fluorescence colors. (Source: J.S. Heslop-Harrison, PhD, University of Leicester, UK)

By mid 2003, the joint effort between Sandia and the University of New Mexico had borne fruit in the form of a prototype hyperspectral scanner and data analysis tools. That, combined with improvements in experimental procedures and design,

made Werner-Washburne able to reliably spot smaller changes in gene expression. "We can get our biological replicate variation down to the level of technical replicate variation, which is about .08 log 2 units for the ratio," she says. "That's awesome. It means that we can believe things that are much less than twofold changes," she says.

There are other prototype hyperspectral imaging systems being developed for biological use and microarray scanning. Beginning with a meeting in 2001, a team of astrophysicists and biologists at the University of Leicester, UK, produced a system that makes use of some advanced, nontraditional detector technology.

Scientists at the University of Texas Southwestern Medical Center, Dallas, also have their own hyperspectral imager, the result of a project that began in the mid-1990s. And, there's a commercial microarray hyperspectral scanner offered by Princeton, N.J.-based Orchid Biosciences Inc.

Spotting impurities

As the Sandia/New Mexico team noted in a 2004 paper [Sinclair *et al.*, *Applied Optics*, vol. 43, no. 10, pp. 2079-2088 (2004)], current microarray scanners measure the total fluorescence emission across a spectral band. These scanners use a bandpass filter, an optical component that passes along only those photons within a specific wavelength range. In a given experiment, some of the photons that make it through the filter are from fluorochromes tagging a gene of interest. Others are from any other fluorochromes present as well as background and contaminating fluorescence.

With a bandpass filter, different fluorochromes applied simultaneously can drown each other out if their emission peaks are too close together. The only solution is to minimize spectral overlap. "Everything has to be widely separated or excited by different lasers," says Haaland.

In their system, the Sandia group used several hardware innovations. One is a Powell lens, an optical component that looks something like a prism with a rounded top. That lens converts an incoming laser beam into an outgoing line of light. The line illuminates the sample and the output along the line is sent through a spectrograph, which splits the emission into its components. These are then captured using a CCD detector enhanced with on-chip electron multi-

plication. After the output along the line has been captured, the line is scanned across the sample. The result is a data cube, with x and y representing the location of a pixel on the slide and the z axis representing the spectral output of that pixel.

The hyperspectral system has proven useful in identifying and removing fluorescence noise sources. Noise can confound microarray results as pointed out by the Sandia/New Mexico group [Martinez *et al.*, *Nucleic Acids Research*, vol. 31, no. 4, e18 (2003)]. The contribution of noise due to contamination is difficult or impossible to pick up with a bandpass filter. If such noise contributes significantly to the overall intensity of a given spot, this can distort experimental results and swamp small changes in gene expression.

Typically this background noise is accounted for by subtracting fluorescence intensity outside a spot from that inside. Hidden in this approach is the assumption that the background is uniform and not localized within the spot itself. Such homogeneity, the researchers have found, is not the case.

"You're really in trouble if it varies over the slide and that's what we've always found," says Werner-Washburne. "None of the spots are the same in terms of that spot-localized fluorescence, the contaminating stuff."

The Sandia and New Mexico researchers compared an Axon 4000B bandpass filter-based commercial scanner with their hyperspectral imaging system and its multivariate curve resolution for Cy3-labeled cDNA. By separating the contamination from the actual fluorochrome emission, they were able to show that 75% of spots on some slides were off in green channel intensity by more than a factor of two and 25% were in error by a factor of 4.5. In an actual experiment, these contamination-driven errors would skew the gene expression results.

Eye on the stars

Another hyperspectral prototype comes from the University of Leicester. For this system, astrophysicists teamed up with biologists to create an imager based on a new detector, a superconducting tunnel junction camera (STJC). This collaboration is not as surprising as it seems at first because the two groups have many of the same goals, although at different magnifications. Stars, like fluorochromes, are point sources of light. Astronomers, like biologists, work with few photons and must tease information out of them.

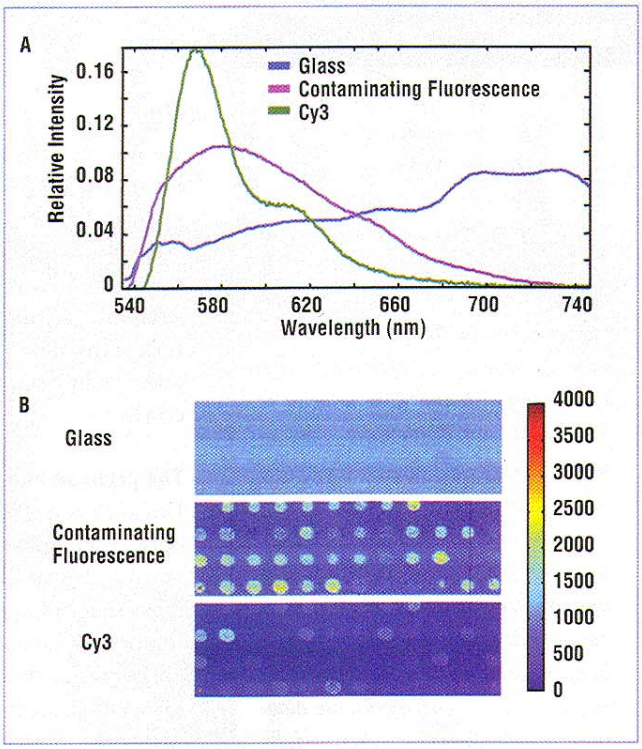
"The sensitivity in detecting very small signals is the same," says Leicester molecular cytogenetics lecturer Trude Schwarzacher, PhD, in speaking of the two scientific disciplines. Another similarity, she adds, is the need to "...try to figure out what the composition is of the light which you're receiving."

Leicester detector physics professor George Fraser, PhD, recalls that despite this commonality in requirements there hadn't been much progress in the collaboration, until a lunchtime meeting that took place in 2001. That's when Fraser decided to take a different approach.

"Instead of saying 'Hi, biologists, I have the solution to your problem. By the way, what is your problem?' We went the much more productive route of sitting down, saying, 'Look, what kind of detection problems do you have?'" Fraser says.

Leicester biology professor J.S. Heslop-Harrison, PhD, was present at the meeting. When he described what he needed for gene analysis, Fraser suggested they use the STJC. Originally developed for astronomy, an STJC operates at 300 milliKelvins or below. At this temperature, helium is a solid and electrons team up in Cooper pairs. When a photon barrels into such a couple, it splits the pairs apart. Because of an applied magnetic field within the camera and the magic of quantum mechanics, these free electrons tunnel from one side to the other of an aluminum oxide insulating layer at the rate of 5,000 to 10,000 per photon.

"The number that it splits is exactly proportional to the color or the energy of that photon," says Heslop-Harrison. Thus, there's no need for gratings, prisms, spectrographs, or any of the other techniques that make fundamen-



Hyperspectral scan results of a hybridized Corning preprinted array show the impact of contaminating fluorescence. Researchers at Sandia and the University of New Mexico scanned Cy3- and Cy5-cDNA hybridized Corning preprinted DNA microarrays with their hyperspectral imaging prototype and an Axon 4000B scanners at 10 microns spatial resolution. (A) Emission spectra of fluorescent species, normalized to unit length. There is green channel overlap near the Cy3 emission peak. (B) Corresponding concentration maps of fluorescent species as determined by hyperspectral imaging and data analysis. (Source: David Haaland, PhD, Sandia National Laboratories)

Glimpsing the Future

Instead of seeing the world through a filter-based window as is done with commercial scanners, hyperspectral imaging systems capture all of the visible emission spectra for every pixel in a microarray. "It actually collects the photons from the entire spectrum. So you actually get more from each component," says University of New Mexico biologist Maggie Werner-Washburne. "You're getting that whole thing, so it's got the potential to be more sensitive."

University of Leicester biology professor J.S. Heslop-Harrison says that greater sensitivity will pay dividends. For example, a hyperspectral imager based on new detector technology would speed up disease diagnosis. He says when fully commercialized the method will "... let us increase throughput between 100 and 1,000 times."

tally monochrome CCDs, films, and photomultiplier tubes able to see color.

After several years of work, the Leicester group developed an STJC-based prototype [Fraser *et al.*, *Review of Scientific Instruments*, vol. 74, no. 9, pp. 4140-4144 (2003)]. The researchers reported that their hyperspectral imager offers a wavelength resolving power of about 10 and considerably improved performance over other alternatives. "We can get sensitivity and speed of analysis or resolution which is about a thousand times better than that for a photomultiplier system that would be the current-state-of-the art for DNA hybridization and microarray type hybridization analyses," says Heslop-Harrison.

On the other hand, the STJC approach does call for an operating temperature that is currently both difficult and expensive to achieve. Presently, this cooling is done using helium dilution refrigerators. These coolers are bulky and require a helium supply. According to Fraser, such refrigerators are not suitable for remote astronomy locations or biology labs. However, he says an alternative is becoming commercially available. The new technology is based on paramagnetic salts and adiabatic demagnetization refrigerators. This new approach promises to make achieving the temperature less costly.

The promise and the peril

David Piston, PhD, molecular physiologist and biophysicist, Vandervilt University, Nashville, Tenn., welcomes hyperspectral imaging—with one important caveat. Piston is primarily concerned with multicolored green fluorescent protein (GFP) imaging, but his position is perhaps applicable to other life science research. The promise of hyperspectral imaging and multiple fluorochromes, according to Piston, is that, in his field of research, most possible single combinations have already been looked at. He says, "The trick is now to start looking at multiple things and look at the dynamics between them."

Currently, Piston uses a commercial confocal microscopy system, the Zeiss LSM 510 META, to regularly image three fluorochromes. He's done as many as six at a time. Using more fluorochromes allows interactions to be probed. For example, Piston says that tracking two proteins that both translocate upon cell stimulation requires labeling the proteins and cell organelles. That requires three fluorochromes. Otherwise, researchers cannot tell if the proteins translocate to the same place within the cell or not.

However, Piston says that many biological specimens operate on the verge of fluorescent saturation. So it is not possible to replace photons lost along the way due to diffraction gratings or other optical elements that split a fluorescent spectrum into its component parts. A bandpass filter, on the other hand, will let anything through. In Piston's view, making every photon tell a tale has to be balanced against the reality that every photon is precious.

"As long as you're not losing photons, then you might as well get as much information about them as possible. But when you start losing photons, you better watch out," Piston says. If this happens it must be clear that "the additional spectral information is worth the signal shortfall, a deficit that can impact sensitivity or throughput."

"The key is for the systems to generate very high throughput," says Piston. "There certainly are some systems that use words like hyperspectral that are very, very low throughput. They're almost useless for the things we do."

The desire to minimize photon loss and so maintain sensitivity and throughput is one reason why the Leicester group is pursuing the STJC. Sandia's Haaland says his system has optimized optical throughput via careful design and a highly sensitive detector.

A key difference between some current commercial hyperspectral systems and the academic prototypes is the number of channels. The Zeiss product, for example, can capture eight out of 32 channels simultaneously whereas the prototype imagers can capture hundreds. Thus they might offer biologists a better tool.

"The more accurate your instrument is, the more truly you're going to see what happens. You've got to distinguish between the variation an instrument can give you and the variation which is present in nature," Leicester's Schwarzacher says.

Hank Hogan is a freelance writer based in Austin, Texas.

For more on the organizations mentioned here, refer to this article at www.genpromag.com