Special News Feature

What Lies Beneath: In Vivo Stem Cell Imaging

Illustration credit: Tavares Jones.

No other branch of science is quite so laden with expectation. But while corralling stem cells for viable therapies, researchers have found themselves asking an entirely separate question: How do we track them?

Stem cells. Call them the great translucent hope. Between clinical trials, legal challenges, and endless media reports, perhaps no other branch of science is quite so laden with expectation. For the general public, stem cells are filtered largely through the lens of hyperbole, with stem cell therapies touted as a sort of scientific second coming. For the researchers who study them, expectations are more tempered, as are the timelines for possible medical advances. Yet there's no denying the excitement; these cells represent a drug development platform, a window into the pathogenesis of disease, and of course, a potential source of transplantable therapies.

In the pursuit of implantable stem cells, though, one question often goes unanswered: Once researchers get stem cells into a body, how will they track them?

It's a question that goes to the heart of stem cell-based therapeutics. Stem cells per se are not much use therapeutically; it's what they change into—cardiomyocytes and islets and neurons, for example—that counts. Or, as James Mansfield, director of tissue analysis applications at Caliper Life Sciences, puts it, "When [stem cells] grow up, what do they become?"

Caliper is one of the companies developing technologies to address such questions. The firm (which in December

acquired rival company Cambridge Research & Instrumentation) sells both fluorescence- and bioluminescence-based whole-body imagers for small animals. These imagers—basically ultrasensitive cameras in very dark boxes—enable researchers to peer past skin, tissue, and bone to visualize the stem cells within. But there are other imaging modalities too, including the acronym-rich toolset of the healthcare industry: CT, MRI,

Snapshot of the zebrafish digital embryo with a view on the closing blastopore. The colored spheres indicate nuclear positions in the entire embryo at 12 hours post-fertilization. Nuclear positions were automatically detected from a DSLM time-lapse recording of a histone-eGFP labeled wild-type embryo. Colors encode local average movement speeds (cyan for orange, 0–1.2 $\mu\text{m}/\text{min}$). Courtesy of Philipp Keller.

PET, and SPECT. At the moment, none of these approaches is perfect; the general consensus seems to support a multimodal approach integrating multiple orthogonal methods. New techniques are in development. In the meantime, stem cell scientists are making do with a rich and ever-growing in vivo imaging toolset.

Translational Imaging

For those researchers interested in what stem cells are doing in the body, in vivo imaging offers several obvious advantages over slide- and plate-based methods. Cells behave differently—and less physiologically—in culture than in live organisms, for one thing. In vivo experiments are also cleaner, according to Stanford University cardiologist Joseph Wu: researchers can image a single test subject repeatedly over time, instead of sacrificing several lab animals at each of several time points and then trying to make sense of the resulting, aggregated data. "There's a lot of variation from animal to animal," Wu says.

That said, in vivo imaging isn't easy, and can be bedeviled by issues like signal penetration, scatter, resolution, and sensitivity. Stem cells add additional complexities, says Mansfield, in that they are relatively rare. "Stem cells are a small population," he says. "Even if you inject a million cells, it's not that many

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Labeling process of human mesenchymal stem cells. (Left) Stem cells are centrifuged after incubation with iron oxide nanoparticles (FDA-approved contrast agent for MR imaging). (Center) The labeled cells are washed to remove cell debris and free contrast agent via repeated centrifugation procedures and supernatant removal. (Right) The pellet of successfully labeled stem cells shows a brown stain. These cells are now ready for transplantation into cartilage defects for cartilage repair. Courtesy of Heike Daldrop-Link.

when spread throughout the animal.” Couple that with the fact that the tissues researchers care about often are deeply buried, and it becomes even more difficult to track them down.

Heike Daldrop-Link, associate professor of radiology at the Stanford University School of Medicine and member of the Molecular Imaging Program at Stanford (MIPS), concentrates her research on techniques for in vivo imaging of stem cells and cancer cells. But not just any imaging modality will do. As an M.D.-Ph.D. who splits her time between the lab and the clinic, Daldrop-Link chooses methods with “very high translational potential.”

Her imaging modality of choice is magnetic resonance imaging (MRI). “Traditional MRI manipulates hydrogen nuclei in fluid under a static magnetic field to obtain regional contrasts based on differences in proton density, flow, or biochemical structure,” explains one 2007 review of cardiac stem cell tracking. To be tracked in infarcted myocardium, stem cells need to be enriched with a contrast agent that produces a sufficient positive or negative signal to distinguish them from the background (1).

The contrast agent Daldrop-Link favors is superparamagnetic iron oxide nanoparticles (SPIO), which happens to be an FDA-approved treatment for iron deficiency called ferumoxytol. Her lab uses SPIO to study the survival of stem cell implants in a rat model of arthritis. Specifically, they load about 75,000 human bone marrow-derived mesenchymal stem cells with SPIO, and then inject them into small holes drilled into the knees of rats.

Although it isn’t possible to view individual cells by MRI, the technique does give Daldrop-Link a general sense of implant viability. Iron oxide nanoparticles appear hypointense (that is, dark) under certain MR conditions, called T2-weighted images. In living cells, she says, the iron is slowly metabolized, producing a signature

that decays consistently over time. Dying cells, though, release their iron into the environment, producing a stronger, darker signal. And because the imaging is done without sacrificing the animal, they can make those measurements repeatedly over time; according to Daldrop-Link’s lab manager, Rosalinda Castaneda, who performs some of these experiments, labeled cells are visible for at least three weeks post-transplantation, and possibly longer.

Dara Kraitchman, associate professor of radiology at Johns Hopkins School of Medicine, is another stem cell researcher taking advantage of MRI. Kraitchman is exploring transplantation of bone marrow-derived mesenchymal stem cells in a rabbit model of peripheral artery disease. To protect those cells from the recipient’s immune system, her team encapsulates the cells inside alginate, which is porous enough to allow growth factors out, but not so much to allow immune cells in.

The capsules offer another advantage, as well. “If you put too much of anything inside a cell, it’s going to be toxic,” Kraitchman says. Instead of overloading the cells with contrast agent, Kraitchman loads the surrounding capsules with perfluorooctylbromide (PFOB), a multi-modal contrast agent. PFOB’s fluorine atoms are MR-active, while its bromine atom is radioopaque (making it visible to X-rays). Her team can thus visualize the

Lung tumor model. H460 cells implanted orthotopically expressing luciferase coregistered with uCT on the Quantum FX. Courtesy of Caliper Life Sciences.

Full-body shots of a medaka juvenile. The 10-day-old specimen was recorded from multiple views with a digital scanned laser light sheet fluorescence microscope (DSLM). The acetylated tubulin immuno-staining shows the developing brain and spinal cord of the approximately 5 mm-long fish juvenile. Courtesy of Philipp Keller.

capsules with fluorine MRI, CT scans, and even ultrasound—thereby enabling, for instance, ultrasound-guided delivery and post-delivery MRI tracking.

According to Kraitchman, implanting cells within alginate capsules increases their survival time substantially. Most implanted cells die within 24 hours, she says, and fewer than 5% will survive a week. “We have data that shows that, when you put them in these bubbles, at two weeks 70–75% of the cells are still surviving.”

Two Labeling Approaches

Fundamentally, there are two approaches to in vivo imaging, says Stanford’s Wu. One, exemplified by Kraitchman and Daldrop-Link, is to physically label cells with an indicator reagent of some sort; the other takes advantage of reporter genes such as GFP or luciferase.

The advantage of the former approach, Wu says, is simplicity. It’s easy to load a cell with contrast agents, fluorescent dyes, and the like. The disadvantage? “You can’t really track the cells for a long period of time,” he says, because the dye dissipates as the cells divide and die. Physical dyes also don’t care whether cells are alive or dead.

With reporter genes, in contrast, researchers never need worry that their imaging reagent will become diluted or transferred to other cells; only live, genetically modified cells will be labeled, and so too will their daughter cells. Thus, as implanted cells divide, the resulting signal actually increases. As an added bonus, reporter gene expression can be either

constitutive or inducible, or even timed to kick in at a given developmental stage. But, the technique’s reliance on genetic modification could limit its translational potential.

According to Wu, reporter genes are compatible with a wide range of imaging modalities. There are GFP and luciferase of course, but there’s also herpes simplex virus thymidine kinase for PET imaging, transferrin receptor for MRI, and the Na/I symporter for SPECT. In collaboration with Northwestern University investigator Thomas Meade, Daldrop-Link’s lab uses a beta-galactosidase reporter to activate an injectable gadolinium-based MRI contrast agent called EgadMe, which is inactive unless acted upon by beta-galactosidase.

Of all these, luciferase is probably the most sensitive, says Wu; it can detect anywhere from 100 to 500 cells. By comparison, PET offers better anatomic detail, but much poorer sensitivity (“500,000 cells or so,” he says). But bioluminescence is impractical outside of rats and mice since larger animals simply are too big for the light to reach the detector efficiently.

Stacey Cromer Berman, a graduate student at the Johns Hopkins School of Medicine, is studying transplantation of neural stem cells. She uses both physical and reporter-based methods, combining BioPAL’s Molday ION Rhodamine B SPIO with a luciferase reporter so she can assess both the location and viability of transplanted cells. (The former she detects using a 9.4-Tesla small animal MRI; the latter, a Xenogen IVIS Spectrum.) Furthermore,

because Molday ION is fluorescently tagged with Rhodamine B, Cromer Berman can also locate the particles post-mortem, on histochemical slides. “We can compare where the nanoparticle was by fluorescence to what we see on the MR images,” she says.

Bioluminescence is Wu’s bread and butter. His team uses complex multigene reporters containing both fluorescent proteins and luciferase to study human embryonic stem cell biology. In one experiment, Wu’s team transfected these cells with genes for luciferase (for bioluminescent imaging), a fluorescent protein (for flow cytometric purification of transfected cells), and HSV-thymidine kinase. When they injected these cells into the shoulder of a mouse, the undifferentiated hESCs began to grow, blooming on bioluminescent images into a cellular supernova of blue and green and red—a teratoma. Subsequent administration of the drug gancyclovir, which targets HSV-thymidine kinase, provided a fail-safe kill switch for the runaway transplant.

“In this case,” Wu wrote in 2010, “reporter gene imaging not only monitored teratoma development, but also served as part of the therapeutic intervention against cellular misbehavior” (2).

Through The Bone Brightly

Charles Lin, of the Wellman Center for Photomedicine at Massachusetts General Hospital, uses fluorescence in his stem cell imaging studies, as well.

Optical microscopy, Lin says, “is the only technique that has single-cell sensitivity and resolution.” But there is no simple combination of markers that can reliably light up stem cells. “That has been the major shortcoming of everything we do,” he concedes. Because of this, Lin eschews fluorescent proteins. Instead, he labels bone marrow–derived hematopoietic stem cells with a lipophilic dye called DiD (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate), injects them into the tail veins of mice, and then scans the recipients’ skulls to find those that took up residence in the marrow.

Over the past decade or so, Lin’s lab has spent some \$300,000–400,000 designing and building a homemade microscopy system that combines confocal and two-photon imaging to peer within the bones of the skull, capturing multicolor optical slices at the interior marrow–bone interface up to a depth of about 200 microns. (The skull itself is 400–500 microns thick, he says, whereas long bones, traditionally associated with hematopoiesis, are far thicker.) “That allows us to look beyond the surface of the bone, deeper into the bone, and not have something in front of it obscure our image.” In a typical experiment, he simultaneously visualizes the injected labeled stem cells, blood vessels (labeled with a near infrared quantum dot), osteoblasts (genetically labeled with GFP), and the collagen of the bone itself (which produces a so-called “second harmonic signal” without exogenous labeling).

Using this technique, Lin has helped clarify confusion regarding stem cell niches within the bone. Previously, researchers believed there were two separate niches, one rich in endothelial cells and one rich in osteoblasts, and that the cells migrated between them. “Our work shows that the cells don’t have to migrate at all,” he says, “because they are at the same place.”

Lin’s is just one of many new microscopy systems in development that can aid in vivo imaging of stem cells. At the Howard Hughes Medical Institute’s Janelia Farm Research Campus, fellow Philipp Keller has redesigned from scratch the light sheet microscopy system he first perfected as a postdoc at the European Molecular Biology Laboratory, which uses uniform but relatively low-level illumination to drive long-term in vivo imaging of developing embryos. Though he hasn’t turned this system loose on stem cells per se, he has used it to create “digital embryos” of the early development of zebrafish and *Drosophila*—developmental periods that include

stem cell differentiation processes. To do that, he records the movement of each cell via a fluorescent protein–histone fusion, like a genetically encoded DAPI. The current system, Keller says, features greater resolution, signal-to-noise, and speed. It is “more than an order of magnitude faster than the old one,” he says, and can reconstruct cell behavior comprehensively for entire complex organisms.

Meanwhile, at the University of California, Irvine, senior scientist Shengwen Calvin Li and biomedical engineering professor Zhongping Chen have devised a microendoscope probe for in vivo, single cell–resolution, 360-degree multiphoton imaging of fluorescent stem cells.

According to Chen, who estimates he has spent more than three years and \$1 million developing this system, the biggest challenge was developing a compact and stable fiber-optic femtosecond laser for multiphoton imaging and miniaturizing the scanning probe needed to use it in vivo. Now, says Chen, the trick will be to make the laser—currently set to about 1050 nm—tunable.

In a 2010 review, Li and Chen laid out seven criteria for what they call a “biological global positioning system,” including single-cell sensitivity, real-time positioning, inducibility, retractability, and the power to track cell viability proliferation and differentiation state in vivo (3). No existing imaging modality meets all those criteria, the authors noted, and neither, says Chen, does his new microendoscopy system.

“It’s just fitting one piece of the puzzle,” Chen says. “All the imaging modalities are complementary.”

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