Microscopy lights up stem cells in action

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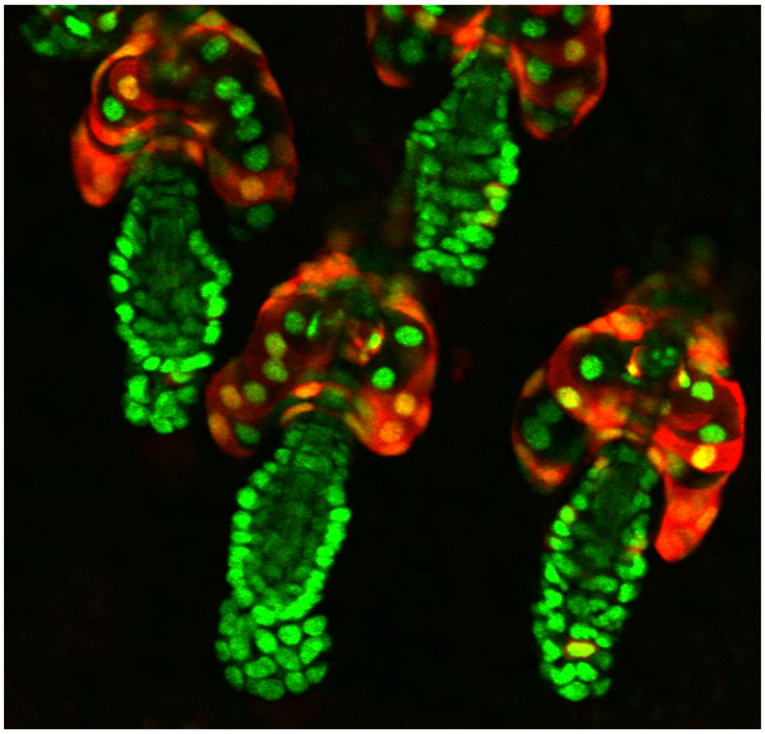
As developmental and cell biologist Valentina Greco stood on a stage in Philadelphia last December, candid time-lapse shots of some of the body's most elusive cells flashed behind her in quick succession. These stem cells, photographed in a living mouse, were caught in the acts of dividing, differentiating, and interacting with neighbors. Such videos are more than just captivating cellular cinema—they could reveal key details about how stem cells work.

Cell biology is in the midst of a microscopy boom, and stem cell researchers in particular are coming up with inventive techniques for capturing live images of these enigmatic cells in their native habitats. Scattered throughout adult tissue, stem cells help maintain tissue function, heal wounds, and in the case of animals such as the zebrafish and axolotl, regenerate entire limbs. Greco, a professor at Yale University, is among the pioneers of using novel combinations of microscopes, fluorescent markers, and sample preparations that light up cellular interactions inside living model organisms to reveal the cues and triggers that prompt these versatile cells to action.

As a microscopist, Greco felt compelled to study the dynamics of stem cell regeneration by watching cells in a living animal. "I followed what I know," she says. "It was easier for me to walk that path than not to." That's not to say it's been easy. She and her team had to focus a high-power microscope on a perfectly still mouse whose cells expressed the right combination of fluorescent markers.

Today, Greco can make time-lapse recordings for up to 18 hours of individual stem cells in an intact living mouse's skin. And she can piece together multiple imaging sessions, repeatedly homing in on the same cells as they move and change roles over months or even a year.

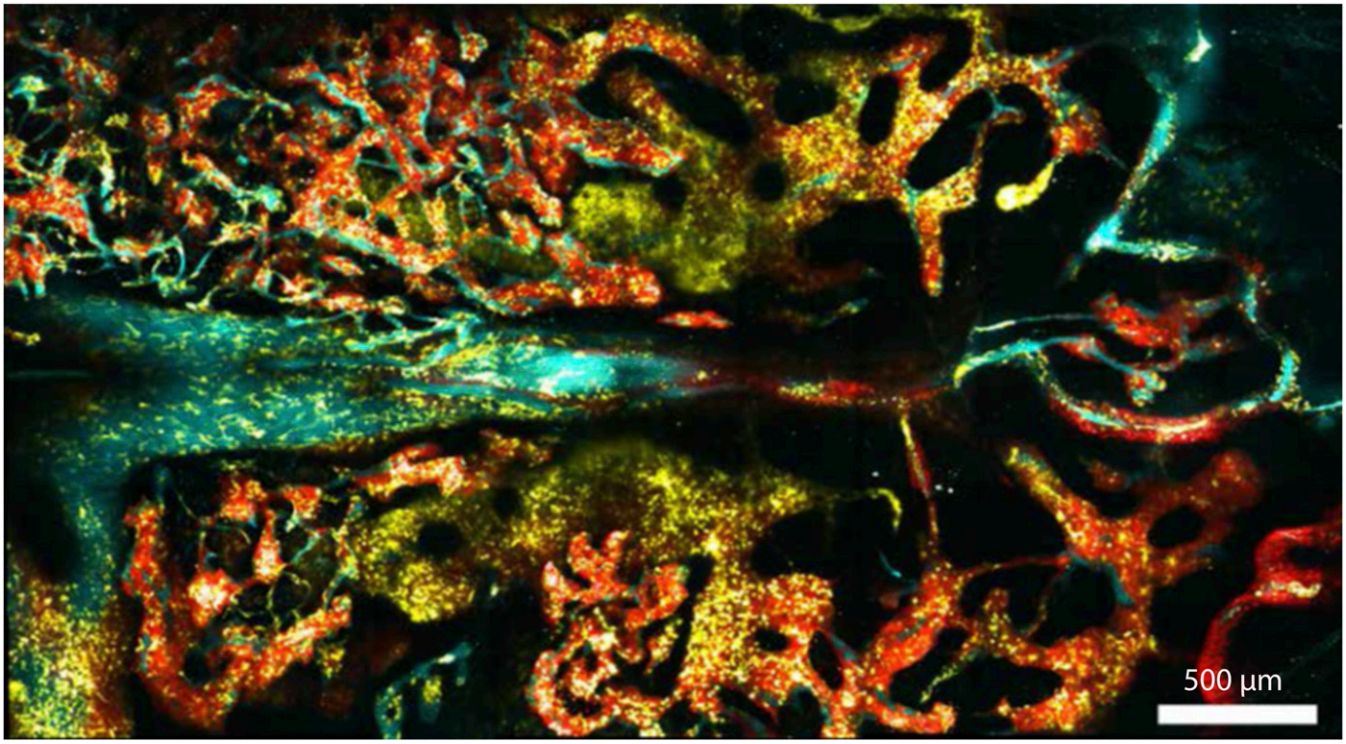


Stem cell biologists are developing microscopy techniques to track individual cells in living animals for weeks or even months at a time. Here, the cells in a mouse's hair follicles glow under a scope operated by Valentina Greco's team at Yale University. Image courtesy of Panteleimon Rompolas (University of Pennsylvania, Philadelphia).

In Living Color

Researchers can catch glimpses of stem cell activity by focusing their scopes on cells cultured in dishes or in tissue extracted from animals. By harvesting regenerating tissue from a series of related mice at different times, researchers can infer how the cells generally behave over a given period. But Greco felt that to understand the factors that drive an individual stem cell's fate—whether it divides, differentiates, sits still, or disappears altogether—she needed to watch that fate play out within a living mouse.

She knew that cell biologist Cristina Lo Celso of Imperial College London had developed a technique for live imaging of hematopoietic stem cells—those that give rise to blood cells—in anesthetized mice (1). Lo Celso removes a small piece of scalp and then looks directly into the bone marrow inside a mouse's skull using a hybrid two-photon and confocal fluorescence microscope. This technique lights up the collagen in bone because of what Lo Celso calls a bit of physics magic. "If you excite the collagen at a specific wavelength," she notes, "then it emits light at half that wavelength, and it's beautiful." Lo Celso also uses



In a mouse model of acute myeloid leukemia, Cristina Lo Celso of Imperial College London used live imaging to show leukemia cells in bright red competing with healthy stem cells and other hematopoietic cells in glowing yellow within fluorescent teal blood vessels. Reproduced with permission from ref. 5.

fluorescent markers that visualize stem cells and other neighboring cells.

Greco studies more easily accessible stem cells in the skin, an organ that regularly regenerates, whether responding to injury or replacing the many millions of cells it sheds every day. She also studies stem cells in the hair follicle, a sort of mini-organ that undergoes cycles of regeneration as hair grows.

Following Lo Celso's lead, Greco set out to track these cells with live imaging. She selected a two-photon microscope and so-called "reporter proteins" or "markers" that glow when expressed in living cells. With the help of researchers at the Yale School of Medicine's In Vivo Imaging Facility, including facility director Ann Haberman, Greco and her team settled on a marker that lit up only stem cells. But the researchers quickly realized they needed a visible backdrop to guide them to their cellular subjects of interest. Through trial and error, the team came up with combinations of markers, including a green fluorescent protein that, when fused with a histone protein, lights up the nucleus of all epithelial cells.

No Sudden Movements

To image, Greco needed to keep the anesthetized mouse perfectly still. But when she trained the scope on the sleeping mouse's back, movements from breathing and even the heartbeat caused the picture to move in and out of focus. She tried focusing on the tip of the mouse ear instead, which was steadier, but maddeningly uneventful. After spending several weeks waiting for something to happen, she shifted the scope to a portion of the ear that was closer to the head. Finally, she observed stem cells in action.

In the lower region, Greco saw glowing red stem cells in a hair follicle. Moreover, some of these stem cells were clearly jostling past green-tinted differentiated cells as they moved to different niches—spaces defined by the collections of neighboring cells and their signaling. In one study, she could see first-hand that the niche each stem cell occupied helped determine its fate (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3895444/bin/NIHMS518697-supplement-sup_video_5.mov) (2). Stem cells in the hair germ layer (tissue below the hair) regularly differentiated into a range of cell types that make up the growing hair follicle. Meanwhile, most of the stem cells in the bulge (tissue surrounding the hair base) divided or remained inactive (2). By tracking those that remained in the bulge, she found that many of these cells ultimately moved to the hair germ where, in their new niche, they too contributed to the making of differentiated cells during hair growth.

Greco then removed stem cells altogether from either the hair germ or the bulge (2). To the team's amazement, hair growth carried on. They watched as stem cells from the other compartment in the hair follicle, as well as altogether different skin epithelial compartments, filed in to assume the missing stem cells' jobs. "The fact that the stem cells are dispensable, I didn't expect," says Greco.

In recent work, the team tracked individual cells surrounding a tiny wound in a living mouse (3). The researchers watched as stem cells migrated toward

the wound, proliferating as they went. Divisions tended to occur in the direction of the wound. Previous studies suggested that cells could not both spread and proliferate at the same time, so this finding relates not only to wound repair but potentially also to cancer cell metastasis, notes Greco.

A Readymade Map

Part of Greco's challenge was tracking cells for an extended period of time. In one imaging session, Greco's team might snap a photo every 5 minutes to create a time-lapse recording of cell migration or every 2 seconds to capture fine-scale activity in a cell's membrane. But a mouse could never sleep long enough for researchers to follow the full fate of stem cells whose lifespans could be as long as the mouse's own. So Greco's team searched for a way to return to the same animal and find the same cells.

At first, they tattooed the mouse's skin with small dots that helped the researchers orient themselves. But soon they realized that the skin provided all the guidance they needed. "If you really concentrate on the details, you start to identify specific landmarks," says Panteleimon Rompolas, a former postdoctoral fellow in Greco's lab and now a cell biologist at the University of Pennsylvania. With the right fluorescent marker, they could light up all the fine vessels and capillaries in the skin—a readymade map. And they could note the placements of hair follicles, which tend to group together in rows, but never perfectly. "Being able to revisit the intact tissue and find the same location, the same cells, allowed us to do studies that span days, weeks, or even months," says Rompolas.

In one such study, the team checked in on the same individual mouse's skin epidermal stem cells every 2 days for 2 weeks (4). Greco saw that stem cells could either divide into two stem cells, maintaining a pool of stem cells at the basal layer, or directly differentiate as they moved toward the outmost skin layer. The visual data provided insight into how the fate of skin stem cells is determined at the individual cell level. (science.sciencemag.org/content/sci/suppl/2016/05/25/science.aaf7012.DC1/aaf7012s2.mov).

Answers in Plain Sight

Some insights gleaned from live imaging would have been difficult to achieve by any other means. Lo Celso recently discovered that in a mouse model of acute myeloid leukemia, a cancer of the blood and bone marrow, cancer cells damaged blood vessels within the bone marrow, where stem cells reside (5). With that niche destroyed, the stem cells couldn't receive the signals that trigger proper differentiation. In one image, she showed red leukemia cells competing with healthy stem cells and other hematopoietic cells in glowing yellow, all within a network of teal blood vessels. Based on this and other observations, she next showed that by preserving the niche environment with deferoxamine, a drug that increased bone marrow vasculature, she could improve stem cell survival (5).

Shosei Yoshida, a developmental biologist at the National Institute for Basic Biology in Japan, who also inspired Greco's work, used live imaging to study stem cells that give rise to sperm in mice. Curiously, these cells come in two forms—a single cell or a string of up to 16 cells that remain interconnected because of incomplete cell divisions. The predominant view for years was that the interconnected stem cells were committed to differentiate into cells that form sperm. Yoshida filmed the cells by first extracting the testes from inside the mouse's body without cutting off the blood supply. His footage suggests that the strings of stem cells regularly fragment and replenish the stem cell pool (6). "I was totally excited to see them being fragmented for the first time," he says.

But practitioners of in vivo stem cell imaging still face hurdles. Researchers such as Yoshida who study internal organs must gain access to the stem cell action long enough to capture the events they're after—a challenge because those cells are in organs below the skin. "By definition, stem cells are stem cells because they maintain the tissues for a long, long time," says Yoshida. Right now, he can only keep mice anesthetized for 2 to 3 days, and the surgery makes repeated imaging intractable.

Microscopy presents its own suite of issues. Stem cell markers have been used as rather coarse on/off switches, says Lo Celso, even though differentiation is more gradual. And there aren't enough fluorescent reporters to illuminate the many interacting molecular signaling pathways driving stem cell fate. "It is really hard to grasp these large networks and how they act together," she says. "The way forward is to generate more diversified reporters."

Despite the challenges on her chosen path, Greco is now excited to see her group's imaging techniques help move the field forward. "Little by little we kept pushing," she says. "Because we knew the payback would be enormous."

1 Lo Celso C, et al. (2009) Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. *Nature* 457:92–96.

2 Rompolas P, Mesa KR, Greco V (2013) Spatial organization within a niche as a determinant of stem-cell fate. *Nature* 502:513–518.

3 Park S, et al. (2017) Tissue-scale coordination of cellular behaviour promotes epidermal wound repair in live mice. *Nat Cell Biol* 19:155–163.

4 Rompolas P, et al. (2016) Spatiotemporal coordination of stem cell commitment during epidermal homeostasis. *Science* 352:1471–1474.

5 Duarte D, et al. (2018) Inhibition of endosteal vascular niche remodeling rescues hematopoietic stem cell loss in AML. *Cell Stem Cell* 22:64–77.e6.

6 Hara K, et al. (2014) Mouse spermatogenic stem cells continually interconvert between equipotent singly isolated and syncytial states. *Cell Stem Cell* 14:658–672.