

## THE IMMORTAL CHALLENGE

**Reproducibility in life science is a hot button topic at the moment. Jeffrey Perkel examines the ways in which stem cell researchers are dealing with this issue in their work.**

**This image depicts iCell cardiomyocytes labeled for proteins that are critical for cell structure and contraction, and demonstrates that these cells exhibit normal cardiac biology and can be used in basic and applied research.** Credit: Cellular Dynamics International Inc.

Arguably, there is no hotter area in life science research today than induced pluripotent stem (iPS) cells. With applications in basic research, drug discovery, and cell therapeutics, iPS cells—essentially embryonic stem cells without the embryo—have attracted tremendous excitement from academics, funding agencies, and pharmaceutical companies alike. The first clinical trial based on an iPS cell-derived product has launched in Japan, less than a decade removed from Shinya Yamanaka's discovery of the iPS cell process in 2006.

Yet as iPS cell R&D speeds towards the clinic, a nagging question remains: Just how easy is it to replicate findings between labs?

### Reagent dilemma

No one doubts the iPS process itself; thousands of papers have been published on the subject, with lab after lab successfully generating and differentiating iPS cells into everything from cardiomyocytes to neurons.

But the devil, as they say, is in the details. iPS cells are generated by expressing a handful of key gene-regulatory transcription factors in terminally differentiated cells (such as fibroblasts), causing these cells to revert to an embryonic stem cell-like pluripotent state. Researchers have developed many ways to deliver the transcription factors, including lentiviral or Sendai viral vectors, episomal DNAs, mRNA transfection, and self-replicating RNAs. Even small molecules can now be used to induce iPS cell formation. Ultimately, using these approaches should lead to the emergence of pluripotent iPS cell clones in

a few weeks, if all goes well. Yet the definition of “pluripotency” is “pretty crude,” says Emile Nuwaysir, Chief Operations Officer and Vice President at Cellular Dynamics International (CDI), a company specializing in the generation of iPS cells and the terminal cells differentiated from them.

Pluripotency basically describes a cell that can divide indefinitely, expresses certain pluripotency factors, and differentiates into all three germ layers—endoderm, ectoderm, and mesoderm. iPS cells do vary substantially in genetic background, as they are created from different individuals. Thus, they are at least as different as, say, different inbred mouse strains, according to cell researcher Paul Knoepfler, Associate Professor of Cell Biology and Human Anatomy at the University of California, Davis. But even genetically identical lines created from the same individual can differ in transcriptional or epigenomic profiles—a reflection, perhaps, of subtle differences in culture conditions or handling—which is why researchers typically use multiple independent iPS cell clones for internal validation.

Genetics, though, are only one side of the reproducibility coin—cells are exquisitely sensitive to their environment as well. “It’s amazing how incredibly fickle these cells can be,” says Robert Lanza, Chief Scientific Officer of Ocata Therapeutics (formerly Advanced Cell Technology), which is advancing pluripotent stem cell-based therapeutics. Switching lots of serum or growth factor, he says, “can make all the difference in the world.” Indeed, in a 2010 study by the International Stem Cell Initiative, 5 labs tested 10 embryonic stem cell lines (which are very similar to iPS cells) using 8 culture methods. “Of the 8 culture systems, only the control and those based on 2 commer-

cial media, mTeSR1 and STEMPRO, supported maintenance of most cell lines for 10 passages,” the authors reported. [1]

Unreliable reagents are “a major, major, major, major problem,” says Pamela Robey, Acting Scientific Director of the NIH Stem Cell Unit. “And I think [it] is also a major contributor to the fact that people have a hard time sometimes reproducing even their own data.” Stem cell reagents, Robey notes, cost “an arm and a leg,” often have limited shelf lives, and can vary wildly in quality and potency from batch to batch. “Sometimes a milligram [of growth factor] has a biological potency of 1, and sometimes it’s 0.5,” Nuwaysir says. Robey says she had a situation in her lab where she was using a particular antibody and “getting great, great, great results. And then we bought a new batch, and we could not get that lot to work, come hell or high water.

Lanza recalls one incident at Ocata where two sets of researchers using the same cells experienced very different results—all thanks to the packaging of the six-well culture dishes they used. One of the researchers was using individually wrapped plates, which worked; the other used plates bundled in stacks, which didn’t. Both sets of plates were from the same vendor and even had the same lot number. “To this day we have no idea what it was that made the difference,” Lanza says—perhaps one batch was left out in the sun too long. “Who knows?”

To mitigate such variability, companies and cell banks develop extensively detailed standard operating procedures (SOPs) to document and verify every conceivable detail. At Ocata, such documents can run to “hundreds and hundreds” of pages, Lanza says, documenting everything from how to thaw cells



**Robert Lanza, CEO at Ocata Therapeutics, is working to advance pluripotent stem cell-based therapeutics.** Credit: Ocata Therapeutics.

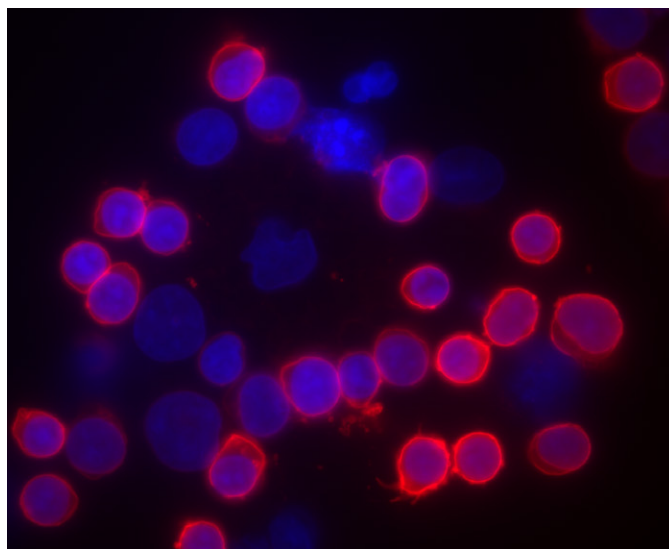
and to how to form embryoid bodies, to the source and quality of the reagents used at each step. “It’s basically getting rid of the variability that exists in a normal research setting.”

It doesn’t help that iPS cell culture is so complicated, with dozens of chemical and physical factors in play. One popular culture medium called TeSR, which was invented in the lab of CDI cofounder (and stem cell pioneer) Jamie Thomson, contains 27 distinct chemical components, Nuwaysir says, “none of which have assays for [biological] potency.” A more recent formulation from Thomson’s lab, Essential-8 (E8), cuts the recipe down to a more manageable eight: four chemicals and four biologicals. “You can have GMP [good manufacturing practice], build assays for them, inventory large quantities for them—E8 is a huge improvement, a much more stable platform,” notes Nuwaysir.

Even if researchers do everything right, stem cells in culture can change or “drift” over time, with “minor clones” taking over the culture and changing its character. As a result, says Glyn Stacey, Director of the UK Stem Cell Bank, his facility routinely tests cells for genetic stability, gene expression, pathogen contamination, and other parameters.

## Enhancing reproducibility

Of course, reproducibility isn’t just a concern for stem cell researchers. There is an increasing emphasis on reproducibility across all of the life sciences, with some journals adopting tighter guidelines for describing reagents and methods in an effort to make the validation of published findings easier.



**Red blood cells generated from human embryonic stem cells.** Credit: Ocata Therapeutics.

This is in part in thanks to a growing recognition that not all data reported in the literature can be replicated. In one high-profile example, C. Glenn Begley and Lee Ellis in 2012 described an attempt to “confirm published findings” from 53 “landmark” hematology/oncology studies. “[S]cientific findings were confirmed in only 6 (11%) cases,” they reported. “Even knowing the limitations of preclinical research, this was a shocking result.” [2]

Still, precisely what those data mean isn’t exactly clear—Begley and Ellis reported their findings in a *Nature* commentary, not a peer-reviewed article, providing no data or experimental details. So it is unclear what the failure rate would be for stem cells. Although Nuwaysir says a similar analysis of stem cell research could yield the same failure rate, he also adds, “there is a logical fallacy to say only 6 of the 50 were true.” Rather, he explains, “it means they were unable to reproduce them. The authors [trying to replicate the studies] have variability too.” Knoepfler, who regularly blogs on the subject of stem cell reproducibility, says, “I expect that the same kind of study done in the stem cell field would yield a much higher rate of reproducibility,” in part, because whereas drug-discovery research is high-risk, “to my knowledge, most stem cell research is already reproduced in multiple labs over time.”

To date, no large-scale studies on the extent of stem-cell data reproducibility have been published. But an example of what such an effort might entail is underway in oncology. Backed by \$1.3 million from the Laura and John Arnold Foundation,



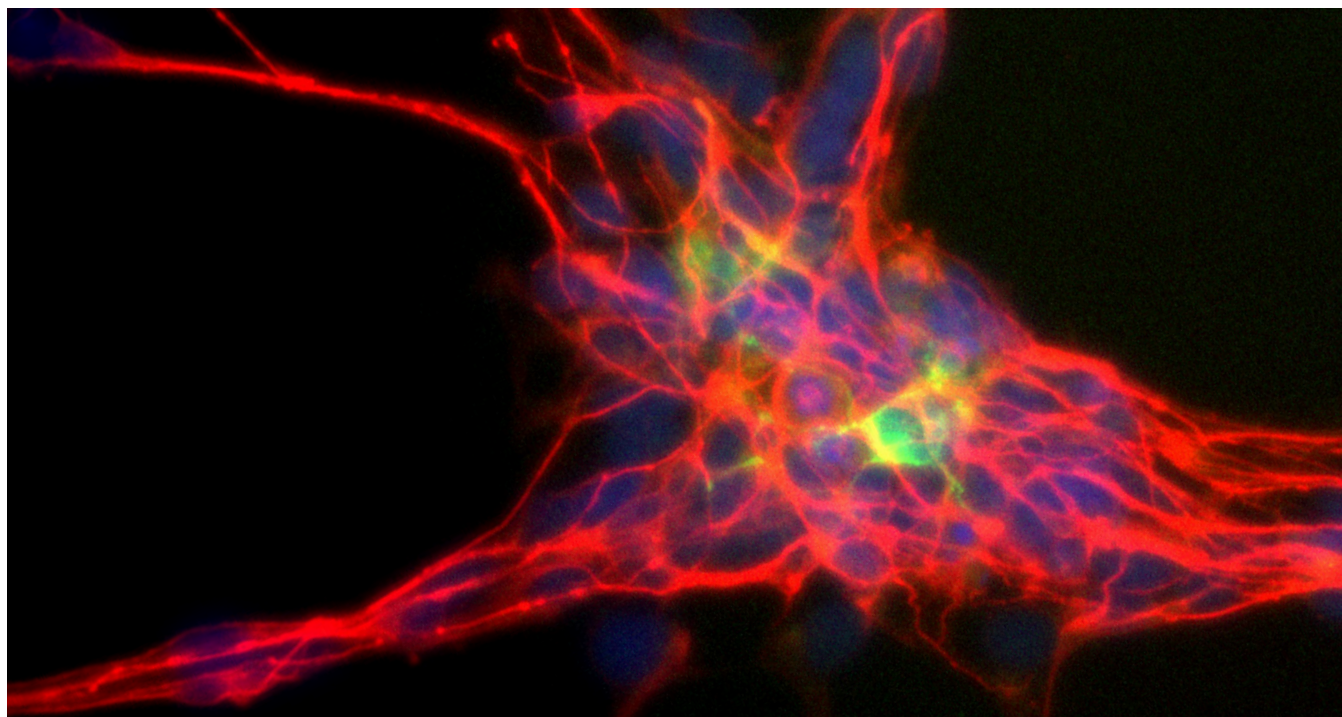
**Emile Nuwaysir says the definition of pluripotency is “pretty crude.”** Credit, Cellular Dynamics International Inc.

the Reproducibility Project: Cancer Biology—a collaboration between the Center for Open Science and Science Exchange—is seeking to validate the key experimental findings of 50 “high-impact” oncology studies published between 2010 and 2012.


According to Elizabeth Iorns, co-founder of the Science Exchange, few papers document the methods used in sufficient detail. “Our experience is that papers rarely have enough information in the protocols to be able to replicate experiments without going back to the authors for help,” she says. Frequently, information is insufficient to uniquely identify the exact reagents used, and methods sections are usually summaries instead of detailed experimental protocols.

The Reproducibility Project has three primary objectives: to generate a public data set of reproducibility; to create a set of best practices for documenting protocols in the literature; and to highlight the value of research into reproducibility. “People undervalue [such studies] at the moment,” she says.

Iorns notes, for instance, that when the now-discredited STAP (stimulus-triggered acquisition of pluripotency) stem cell studies were published in early 2014, many labs rushed to try to replicate the findings. “But it wasn’t actually a replication,” she says. “Many different labs tried different cells and [different] methods and didn’t see the same thing. What does that tell you? How do you interpret that?” It wasn’t until researchers tried to replicate the STAP cell findings precisely—using the same cells and growth conditions—that they could begin to see that the process didn’t work at all.



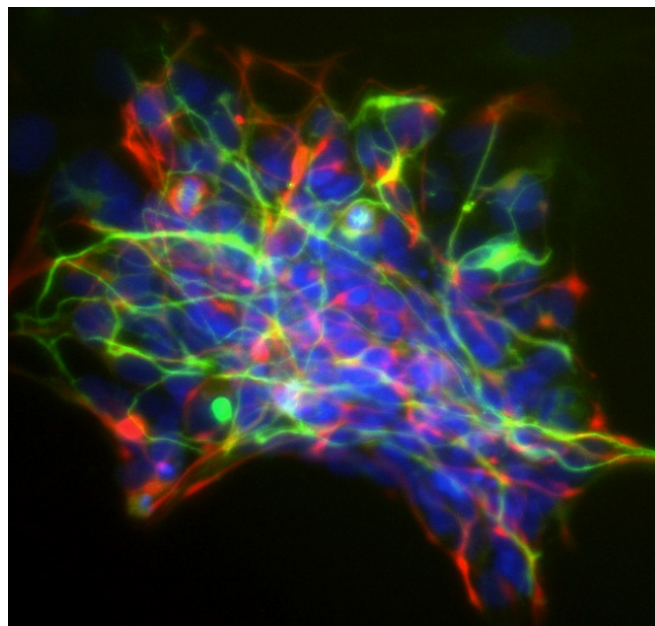
**An induced pluripotent stem cell differentiated into a neural precursor.** Credit: Ocata Therapeutics.

 The Reproducibility Project: Cancer Biology will use, to the best of its ability, “the exact same protocols as the original study,” Iorns says. “We are trying to use the same mouse models, cell lines, and reagents, to understand if you take an experiment from one lab, can you get the same result in another lab?”

## What can you do?

One way that researchers can address stem cell reproducibility is with better communication. For instance, researchers in the lab of Dennis Clegg, Professor and Co-Director of the Center for Stem Cell Biology and Engineering at the University of California, Santa Barbara have developed protocols to differentiate iPS and embryonic stem cells into retinal pigment epithelial cells. When it came time to scale those protocols for use in a clinical trial for the treatment of age-related macular degeneration, Clegg had to transfer the protocol to researchers at the City of Hope.

“You can’t just send a protocol and say ‘do it,’” Clegg says. Stem cell culture, he explains, is “almost an art form.” Researchers need to work closely with their colleagues to learn the nuances, the little details that don’t make it into a printed protocol. “We even in some cases make movies with iPhones on particular [steps].”



**Differentiated human iPS cells.** Credit: Ocata Therapeutics.

Rohit Kulkarni, Senior Investigator at the Joslin Diabetes Center in Boston, is attempting to turn pluripotent stem cells into insulin-producing beta cells. He has been working to replicate recent findings from the laboratories of Doug Melton at Harvard and Timothy Kieffer at the University of British Columbia that purport to have cracked the beta-cell problem. “We are about three-quarters of the way through the Melton and Kieffer protocols, and it’s working so far,” Kulkarni says. “But that’s because we’ve been sure to get the same reagents from the same companies.” Communication was also key. Kieffer’s team uses a technique called “air-liquid interface” culture at one point in their protocol, and Kulkarni’s team had no experience with it. “We needed details on what exactly that entailed.”

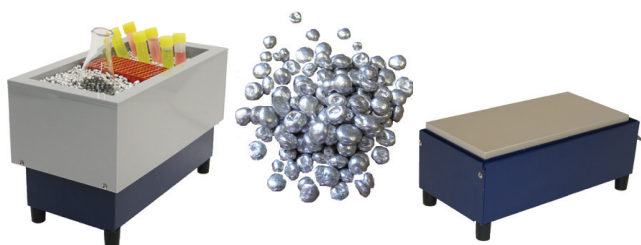
Clegg says his lab has never personally experienced a protocol failing to work as advertised, though he admits he also hasn’t tried too many. But he has experienced first hand the behavioral differences that can exist between seemingly identical cell lines.

One particular embryonic stem cell line, H9, can efficiently differentiate into RPE cells, while another popular line, H1, does not. “What we had heard anecdotally is that H9 tends to be biased towards neural lineages, including RPE, so it sort of made sense,” Clegg says. “But what’s the difference between those two [lines], we don’t know.” Clegg has observed similar plasticity differences between iPS lines—a difference that could pose a problem for clinical applications, especially if they involve patient-specific cells (as opposed to banked and validated HLA-matched iPS cell lines).

One potential solution is to increase the quality and standardization of pluripotent stem cell reagents and methods, and to some extent, that already is happening.

Timothy Kamp, co-director of the Stem Cell and Regenerative Medicine Center at the University of Wisconsin–Madison School of Medicine and Public Health, notes, for instance, that fewer researchers these days culture their pluripotent stem cells on mouse embryonic fibroblast (MEF) feeder cell layers. MEFs, he

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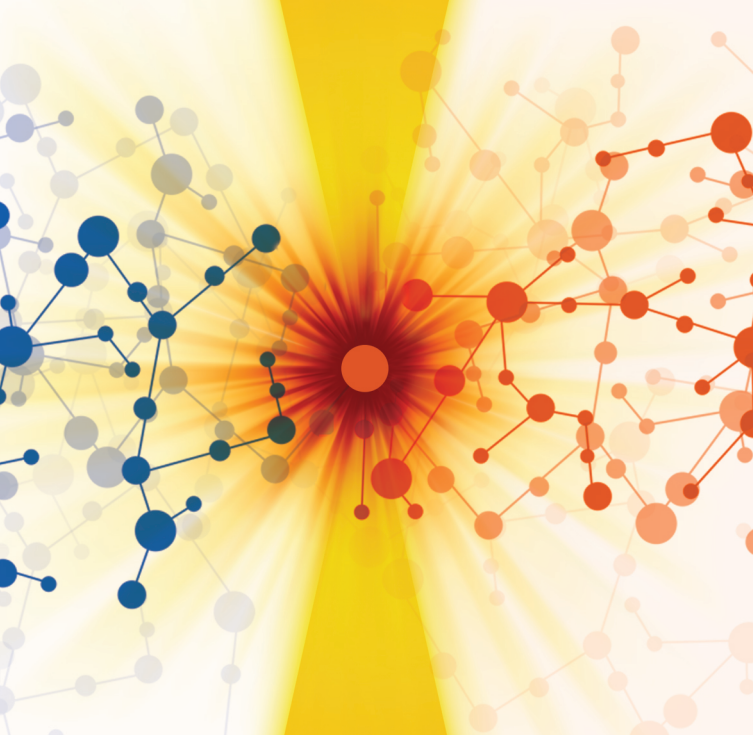
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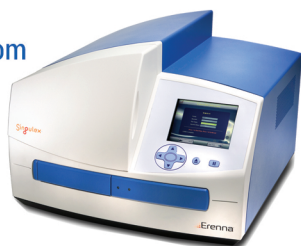
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**Elizabeth Iorns, one of the cofounders of Science Exchange, is involved with the Reproducibility Project.** Credit: Elizabeth Iorns.

explains, made embryonic stem cell culture possible—"They were an essential stepping stone." But they also introduce substantial variability, as each MEF culture is different. As a result, many researchers now use defined substrates, such as laminins and vitronectin, or at least BD Biosciences' Matrigel, a tumor-derived extracellular matrix extract.

Fetal bovine serum as a culture additive is slowly being phased out in favor of better-defined, xenobiotic-free serum replacements. So, too, are protein growth factors, which in a few cases can be replaced with small molecules. In 2012, Kamp coauthored a study showing that human pluripotent stem cells could be differentiated into cardiomyocytes "solely via small molecule modulation of regulatory elements of Wnt/beta-catenin signaling." [3]

Discussions are going on within the stem cell community regarding the development of standard reagents, such as cell lines or nucleic acids, that could be used as stable benchmarks across experiments and between labs. The National Institute of Standards and Technology is also looking into stem cell standards. At the moment though, researchers are mostly on their own. Validation studies aren't sexy, all agree, and nobody wants a PhD in reagent quality control. Yet if stem cell research is to ever reach its full potential, the stem cell community may need to accept that protocol tweaking and experimental validation will be a part of their lives for some time to come.

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