Stem cells: a dish of neurons

Vivien Marx

Labs can generate neurons from pluripotent stem cells to study basic biology and to model disease. Protocols are getting more robust, and labs add personal preferences.

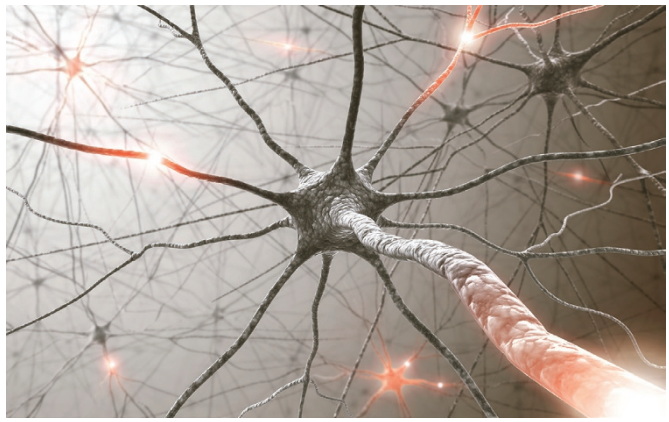
'Depression in a dish' could be a name for a cell culture gone awry. It's also a futuristic notion about modeling human depression in a lab dish, perhaps with serotonin neurons, which were recently shown to be derivable from stem cells. These neurons are an important achievement on the way to that modeling goal, but it will take a wealth of other considerations for labs to reproducibly generate high-quality, functionally mature cells.

To generate neurons *in vitro*, researchers can carefully add transcription factors or chemical cues to adult somatic cells, which directly convert them into neurons in a process called transdifferentiation[1]. A different method involves reprogramming somatic cells into induced pluripotent stem cells (iPSCs), then differentiating those into neurons[1]. Labs can also buy neurons made from iPSCs, although not serotonin neurons just yet.

In the human brain, serotonin neurons have diverse roles including effects on mood, cognition, sleep and appetite, and they have been implicated in conditions such as depression, autism spectrum disorders and anxiety. With access to large numbers of serotonin neurons, researchers could explore the cell biology of these neurons and probe poorly understood questions such as how the release of the neurotransmitter serotonin is regulated, says Patricia Gaspar, a neurobiologist at the Institut du Fer à Moulin, which is part of INSERM, the French Institute of Health and Medical Research. The neurons could be used to study the effect of drugs that target them or to screen for potential new drugs. Labs might assess whether grafting these neurons can treat brain disorders linked to serotonin deficiency.

In vitro–generated neurons can let labs study the functional effects of genetic variation. Imaginably, says Gaspar, one can derive serotonin neurons from cells of individuals with genetic variants of serotonin-related genes and study how the variants influence serotonin metabolism.

A need for better disease models is motivating labs to explore how to generate cells *in vitro*, especially in neurobiology, says Dirk Hockemeyer of the University of California, Berkeley. "There are models to study depression in mice, but that only goes only so far," he says. Rodent models are helpful to neurobiologists, but an animal does not fully recapitulate the human disease state, especially with behavioral and complex disorders.



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Neurons can be grown in the lab. It's not yet a disease in a dish, but labs are exploring how to generate high-quality, functionally mature cells.

Different paths to neurons

Earlier this year, Su-Chun Zhang and colleagues at the University of Wisconsin–Madison published a chemically defined strategy for converting stem cells—human embryonic stem cells and iPSCs—into serotonin neurons by activating Wnt and sonic hedgehog (SHH) signaling pathways[2,3]. The cells' electrophysiological properties were similar to those of serotonin neurons, and when the team tested their response to drugs—a serotonin reuptake inhibitor and a serotonin releaser—the cells reacted in expected ways.

Several labs have explored activating the Wnt pathway with a compound that inhibits glycogen synthase kinase 3 beta in order to convert PSCs into different types of neural progenitor cells. In the Wisconsin lab, researchers used a narrow concentration range of this compound to generate precursors, which became serotonin neurons when the cells were then treated with SHH followed by FGF4. In three weeks, around 60% of the cells were converted to a serotonin neuron subtype, says stem cell biologist Stéphane Nedelec, also at INSERM's Institut du Fer à Moulin, which is why he sees the Zhang lab's work as an efficient approach.

Other approaches have been used to generate these neurons, such as transdifferentiation in which human fibroblast cells are directly converted into neurons[4–6]. Each method has pros and cons, which is why excitement about having serotonin neurons in a dish "will always come with a grain of skepticism," says Gaspar.

More generally speaking, with transdifferentiation, somatic cells—usually skin fibroblasts—take on a neural identity as a result of overexpression of a set of

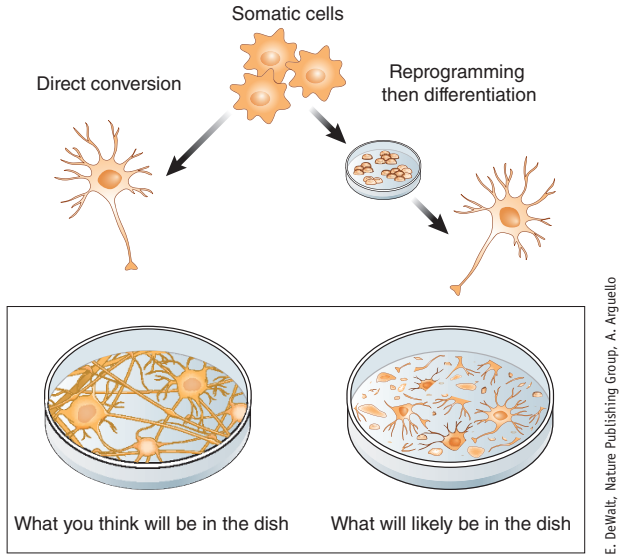
transcription factors that are responsible for neuron specification in embryos. It's cost-effective and rapid in addition to having relatively high efficiency, says Nedelec, and as such it can help scientists study cells from many different patients or be used in small-scale drug screens. But it might not be the method of choice for labs needing large numbers of neurons, he says, because, for example, biopsied fibroblasts can be amplified for only a certain number of passages. The process of transdifferentiation leads directly to postmitotic serotonin neurons, which aren't helpful for studying neurodevelopment, he says. Also, this approach might lead not to subtypes of serotonin neuron, he says, but rather to "ground-state" serotonin neurons.

Direct conversion can lead to neurons with epigenetic marks such as age-related signatures from the cells they were derived from. These might help researchers study late-onset diseases, but they can be a drawback if this signature perturbs the identity, maturation or physiology of the generated neurons, says Nedelec.

In the stepwise approach with reprogramming, in contrast, a gradation of differentiation signals at precise time points is added to the stem cells' environment. Age-related signatures are erased and the cells mimic embryonic development, says Gaspar. A somatic cell becomes an iPSC, then a neural progenitor, then a neuron. The disadvantage is the time it takes to generate, characterize and amplify the iPSCs and then differentiate them. The protocols are more involved than direct conversion, and they might be less robust, which can mean that one lab's work might not be easily reproduced in another, she says. Reprogramming and then differentiating is more likely than direct conversion to produce neuronal subtypes similar to *in vivo* neurons, which can help labs working on physiology and regulation of these neurons or large-scale drug screening. Theoretically, says Gaspar, the iPSCs can be amplified and differentiated in unlimited quantities.

Recapitulating developmental milestones and cues as the Zhang lab has done is attractive, says Ernst Wolvetang, who leads a stem cell engineering group at the University of Queensland in Australia. But beyond the cell lines the lab tested, he wonders how robust this method will turn out to be in a variety of iPSC backgrounds. He and his team see different cell lines react in subtly different ways to small-molecule Wnt agonists.

The directed differentiation approach might be more likely to recapitulate the sophisticated gene expression as well as the epigenetic signatures of *in vivo* cell types, which can also be disease signatures, says Wolvetang. But the weeks and months of differentiation time are frustrating, which lends value to direct conversion.



The yield with *in vitro*-generated neurons can be high, but it can be good to find out what else is in the dish.

A growing number of methods make use of human PSCs to generate various neuronal identities, says Madeline Lancaster at the Medical Research Council Laboratory of Molecular Biology. When making serotonin neurons, the Zhang lab used growth factors and small molecules to mimic embryonic spinal cord differentiation and patterning, which, she says, supports the idea that taking a cue from embryonic development seems to lead to the most effective method. "I think we are seeing more and more of this, and I think it points to the idea that learning more from development will also have important implications for developing differentiation protocols," she says.

The availability of different protocols and tools gives scientists the option to select what is right for them, says Hockemeyer. For studying many monoallelic diseases, directly converted neurons are helpful tools, he says[7]. But labs have to keep in mind that imposing a transcription factor profile onto a fibroblast might not recapitulate the actual development of a patient's neuron.

A question of maturity

Differentiation protocols can get "stuck" and the resulting cells might be embryonic, says Hockemeyer, which is challenging for disease modelers. Even when, for example, *in vitro*–generated serotonin neurons show typical electrophysiological responses, says Wolvetang, labs cannot assume they function as mature cell types. *In vitro* neurons that have been directly converted from somatic cells as well as those made by reprogramming and differentiation can exhibit an immature gene expression signature, he says, which can affect many experiments and drug screens.

Although immature cells can model some pathological features of diseases such as Parkinson's, amyotrophic lateral sclerosis or Alzheimer's, mature cells are needed for studying these conditions, says Michael Yaffe, a scientist at the New York Stem Cell Foundation Research Institute. "It's a big challenge in the field in general to try and obtain mature cell types," he says. Virtually all cell types differentiated from PSCs *in vitro* tend to be functionally immature, he says, including neurons, cardiomyocytes, hepatocytes and pancreatic beta cells.

To analyze how mature their cells are, labs can study gene expression signatures, using, for example, CellNet, a computational platform hosted at Harvard Medical School to score transcriptional profiles; CoNTExT, a computational toolbox from UCLA for matching *in vitro* and *in vivo* transcriptional signatures in brain cells; or Cortecon, a database of transcriptional information of the cortex hosted by the Neural Stem Cell Institute. Researchers have recently applied single-cell RNA sequencing to assess gene expression changes that cells undergo during direct conversion to neurons[8].

The true functionality test is likely to be an *in vivo* one. "I think transplantation will be an important readout to see whether the cells integrate in the proper network and

modulate neural activity in the same manner as endogenous neurons," says Lancaster. These tests might involve a xenograft or transplantation, says Hockemeyer, to see whether *in vitro*–generated cells integrate into the context of typical neighbors. Cells can seem fine in the dish but be dysfunctional in a tissue context.

Patrick Mcmullan

It's a big challenge in the field to obtain mature cell types, says Michael Yaffe.

Organoids, which are self-organizing complex tissue cultures developed from stem cells, offer new modes to graft *in vitro* cells. Organoids "will become the standard or should become the standard of testing of what has been made in a dish," says Hockemeyer.

In 2013, Lancaster and Juergen Knoblich at the Institute of Molecular Biotechnology of the Austrian Academy of Sciences used human PSCs to generate brain-tissue organoids. As Lancaster explains, most effort in brain organoids to date has been placed on forebrain identities. But she and her colleagues have shown that when brain organoids are made without growth factors or small molecules, they generate other identities, including midbrain and hindbrain identities. Potentially, that is an assay opportunity in which to test serotonin neurons. By applying growth factors the way that the Zhang lab did, she says, one might be able to push the identity toward these hindbrain fates and then see the function of serotonin neurons within a 3D context.

When labs differentiate stem cells into neurons, quality control is best built into their process, says Yaffe, to help them later compare results from cell lines derived from different people, to correlate a disease phenotype or to compare cell lines from different studies. A yield of 50–60%, as in the case of the serotonin neurons, "does sound like a good yield" for analysis and characterization, he says.

Part of quality control is knowing "what else" is in the dish, says Yaffe. There might be incompletely differentiated neurons, neuron-related cells or neuronal types other than the intended one. Very subtle experimental aspects can influence yield, such as whether a factor is pipetted directly into the tube or down the side of the tube, and whether it is added slowly or quickly.

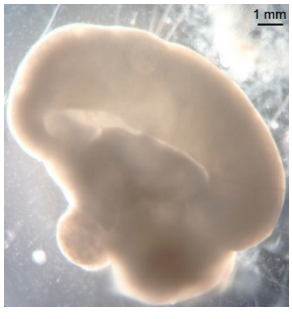
The solution is automation, which, he says, is a self-serving suggestion, given that the foundation's lab is highly automated[9]. But even an automated process has to be fine-tuned to determine, for example, when best to add which factors to the cells. Assays in stem cell biology are typically run in duplicate or triplicate, but labs tend to lack the time and resources for studying many cells, he says. That means that scientists won't always know their cells' typical variability and must hope that what they see is natural heterogeneity, he says, rather than a consequence of the experimental procedure.

Addressing variability

It's a conundrum that the final makeup of cell types in a dish can vary. Scientists might intend to generate one type of neuron but find that some resulting cells are not the intended neuronal cell type, says Wolvetang. Many factors play a role—differing iPSC cell culture methods, varying differentiation techniques, the different time points chosen to analyze the cells—all of which can hinder reproducibility.

iPSCs react differently to the same transcription factors, and results can differ from one experiment to the next, says Hockemeyer. Labs can use transcriptional profiles to control for variability, and he follows the suggested approach from Fred Gage and colleagues at the Salk Institute for Biological Studies: a protocol is acceptable if it does not affect the expression of genes under study.

Labs will probably always use their "favorite" method to generate PSCs and for differentiation, says Hockemeyer. He uses a protocol developed in the lab of Lorenz Studer at Memorial Sloan Kettering Cancer Center by which two SMAD signaling inhibitors make neural induction more efficient. "It's a beautiful protocol," he says, that needs little tweaking, is robust over several cell lines, is well described, and was one of the earliest neural differentiation methods.



MRC LMB

Organoids, which are self-organizing complex tissue cultures developed from stem cells, can be used to test cells generated *in vitro*.

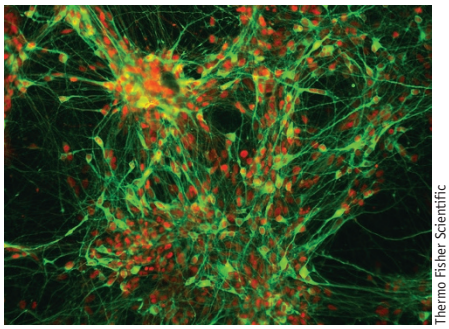
Genetic variability is unavoidable, says Hockemeyer. With inbred mouse strains and cells derived from mouse embryonic stem cells, an introduced mutation is likely to be causative for a phenotype. But given the great degree of genetic variability among human iPSCs and embryonic stem cells, it is harder to declare phenotypic differences. To address this issue, he combines gene editing and stem cell biology. "We generated cell lines that are isogenic except for one locus," he says, by starting with one particular human induced pluripotent cell line as a 'wild type'. Using gene editing to make mutations will lead to phenotypes that can be discerned more easily.

H9 cells are good at neural differentiation, says Wolvetang, but cells from patients will add many sources of variability during the generation of PSCs. Even the different ways in which labs maintain their undifferentiated stem cells can affect how efficacious a protocol is, he says. Guidelines such as those from the International Society for Stem Cell Research, the sharing of cell lines and the existence of protocols "with the nitty-gritty details" may, he says, eventually reduce the level of "frustration in the dish."

Ordering out for neurons

Serotonin neurons might one day be found in sales catalogs, and there is interest in offering them if there are enough labs who need them. Companies currently offer mouse and rat neurons as well as various types of terminally differentiated human neurons. Researchers can also buy neural progenitor cells and use a selection of media or supplements to direct their differentiation and maturation.

Thermo Fisher Scientific offers cryopreserved neurons from rat and mouse, neural progenitor cells from human cell lines such as H9, and a variety of media and supplements to support the differentiation of human neural stem cells. Differentiating stem cells are "like a bar of soap sliding down a ramp," says Alex Hannay, senior product manager for neural cell culture and stem cell differentiation at Thermo. He sees how intently labs heed time windows during which they coax along development. The right factor added



Thermo Fisher Scientific

Markers help to identify cell type or maturation status. Here, FoxA2 (red) and tyrosine hydroxylase (green) indicate that these cells are midbrain dopaminergic neurons.

a little too late won't have the desired effect, he says.

In addition to process, the quality of individual components matters, too, says David Kuninger, the company's senior R&D manager responsible for stem cell culture systems, neurobiology and cell biology. After they license an academic protocol, he and his team characterize the protocol so they can highlight critical moments for users, such as when cells must stay cold or when a quick media change must happen at a particular juncture. The company does alpha-testing of products with a few labs and beta-tests with more labs through an early adopter program.

Kuninger and his team often encounter stem cell labs unwilling to part with their known routine, but this can play tricks in the product test phase. For example, if researchers use cell culture media other than a specified Thermo Fisher product, the cells might die, he says, because one medium can hold pluripotent stem cells in a different state than another. He and his team use such situations to anticipate lab habits and then revise protocols.

Some labs might prefer neural precursor cells because they are mitotic cells, says Hannay. These cells do not look or act like neurons, but they can be induced to a variety of neural fates with a range of products including those from Thermo's acquisition of Gibco, a cell biology company.

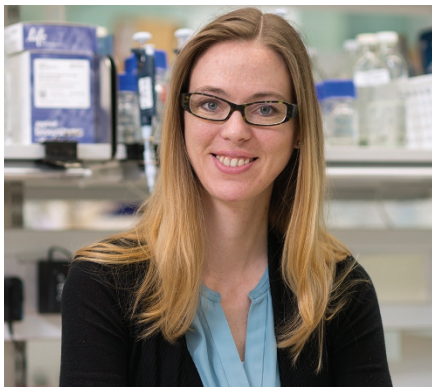
Quality control is also part of the internal process, whether for cryopreserved neurons or media of various types, says Kuninger. The company's teams explore ways to reduce protocol complexity. For example, the company modeled their product for differentiating dopaminergic neurons after one developed in the Studer lab at Memorial Sloan Kettering Cancer Center. It involved eight different media and a 50-day course, which was not scalable, says Hannay, and researchers had to be at that bench throughout the process.

By combining protocol components, Hannay and his team reduced the number of protocol steps and checked gene expression signatures to ensure that the neurons remained unaffected by the change. They also made it possible to interrupt the differentiation protocol at day 16. A lab can bank the cells or share them with other labs.

Making protocols more robust takes away "some of the black magic" that is common in many stem cell labs, says Kuninger. The company tests raw materials, optimizes the timing of component-addition steps, and manufactures according to good manufacturing practice to drive consistency and quality. "We have mechanisms to go back and troubleshoot if there are problems," he says.

Stem cell biology is not a field with standardized methods. An iPSC can be biased toward a particular lineage, says Hannay, which can affect yield when trying to pull these cells to a specific lineage. He and his colleagues explore how to anticipate these issues so he and his team can specifically help labs differentiate iPSCs with predictable behavior.

To support researchers working on Parkinson's disease, StemCell Technologies has used existing protocols to develop modular kits for dopaminergic neuron differentiation and maturation with which labs can turn neural progenitor cells into dopaminergic neurons, says Sam Lloyd-Burton, the company's neuroscience product manager. Given the range of basic and drug-discovery-oriented research on depression, the recently developed serotonin neurons might find users in both of those areas, she says.



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Brain organoids can provide a potential tissue context for testing serotonin neurons, says Madeline Lancaster.

As Vivian Lee, a senior scientist at the company, explains, when the protocol for dopaminergic neurons was first published, the yield was only around 10–20%. Now, as labs optimize the process, the number is moving closer to 60–80%. A mixture of cells in the dish remains. When licensing protocols, the company seeks to turn what works in one lab into a process that many can use, including researchers who may be new to working with neuronal cells, says Lee. She and her colleagues start out by replicating the protocol in-house with different cell lines. They study the yield, simplify the protocol and document key points for the customer literature. They, too, characterize raw materials and check whether components remain stable over time and during shipping.

StemCell Technologies has also ventured into organoids. Adding to its offering of intestinal organoids, the company recently licensed the brain organoid developed by Lancaster and Knoblich. Company scientists are currently optimizing the protocol and plan to launch it for customers in 2017. The protocol has more than 40 ingredients, says Lee, and it is a process they want to simplify and consolidate. Out of the box, the company wants the components to be optimized "to make sure that they work together," says Lloyd-Burton.

When characterizing neurons, researchers first look at morphology, then do gene expression and protein analysis, says Lloyd-Burton. Not infrequently, she says, customers tell her they have generated a "Franken-neuron" in the lab: it looks like a neuron, it expresses the right markers, but it does not behave like a neuron, she says. Often maturation is to blame. To help address such issues, the company recently licensed a neuronal medium from the Gage lab. As Lloyd-Burton explains, this medium, BrainPhys, is akin to the brain's environment, and is specifically designed for the functional maturation of neurons. It leads to a higher proportion of synaptically active neurons in the dish.

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