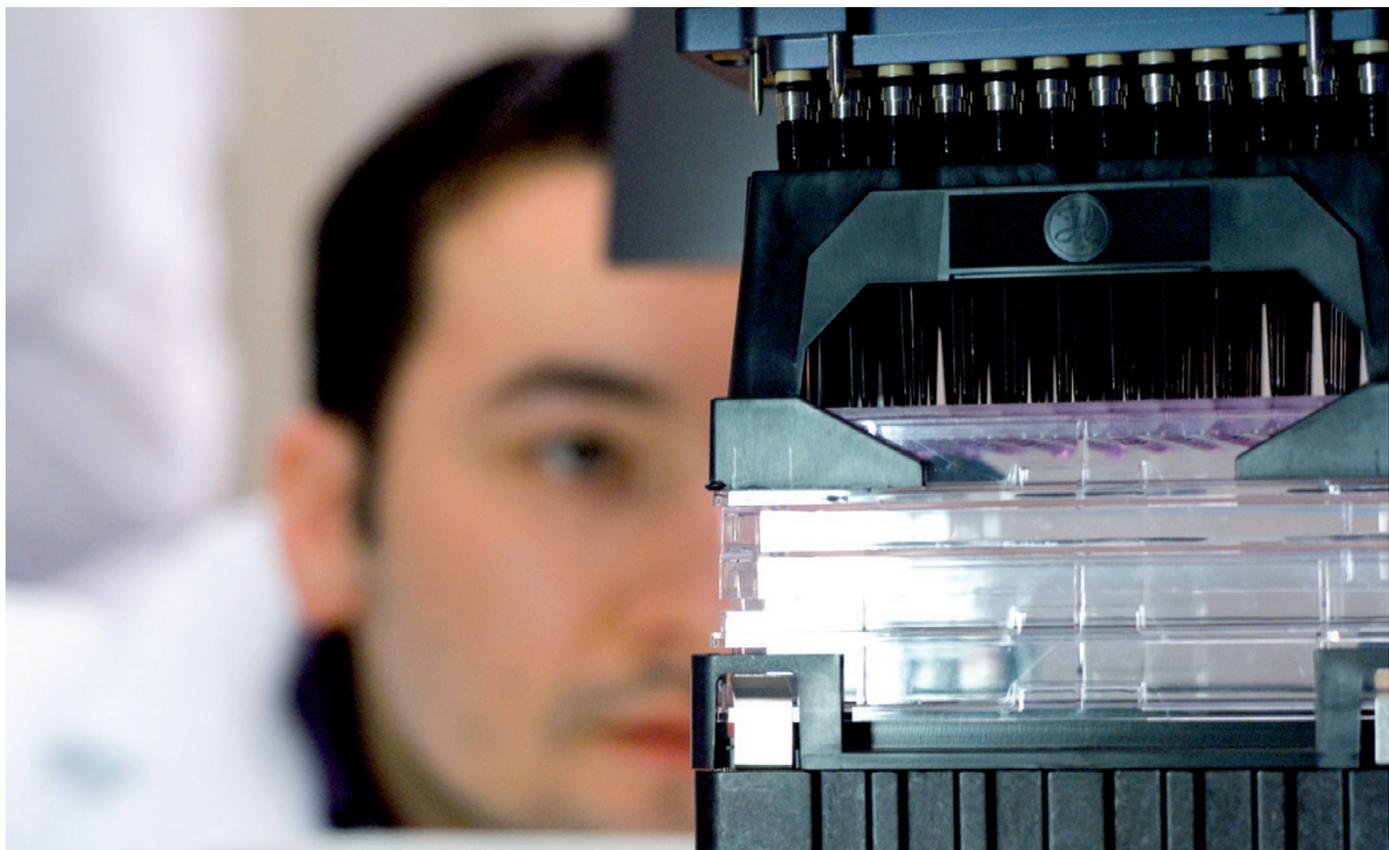


TECHNOLOGY FEATURE

A BETTER BREW

Advances in cell culture media mean that scientists increasingly know what has gone into the mix, and cells are enjoying a more natural environment — even in the lab.



INSPIRO

Scientists explore three-dimensional environments in which they can foster the tissue-like growth of cell clusters.

BY VIVIEN MARX

Cells that thrive in the lab make for happy researchers. And vice versa: biology experiments can grind to a halt if investigators fail to get their cell cultures growing in the right nutrient medium.

That is why the market for such culture media is a lively one, with scores of commercial and home-brewed mixes available to help biologists to deal with all the different cell types that their experiments might require. But although the field of cell culturing can draw on generations of experience, making the right choice of medium is still more of an art than a science.

Even slight differences in media can have a large impact on cells — often for no clear reason. Many scientists mix their own culture media, but that can hamper the reproducibility of scientific findings. John Masters,

an experimental pathologist at University College London and editor of numerous books on animal and human cell culture, says that the recipe for such ‘home-brews’ can be difficult to follow owing to the sheer number of ingredients, as well as variations in purity and content between suppliers, variations between batches from a single supplier, and the difficulties of making relatively small quantities of a labile mixture of chemicals consistently.

However, as scientists come to terms with the importance of knowing exactly what their cells are thriving on, the field is becoming more rigorous. Some researchers, for example, are trying to eliminate culture-media components that originate from animals, because of fears that they could contaminate or infect potential human therapeutics down the line. Other investigators are trying to make growth media reproduce a natural

environment more realistically — for example by creating three-dimensional (3D) tissue structures.

SOME CELLS ARE HARD TO PLEASE

A prime example of the importance of good culture media is in the burgeoning field of induced pluripotent stem cells (iPSCs) — adult cells that have had their molecular clocks turned back to regain the any-fate-is-possible state of their infancy¹. These cells can be redirected to become many cell types, offering prospects for regenerative medicine² using lab-grown tissues to replace or renew aged, injured or diseased tissue in patients.

At the RIKEN Center for Developmental Biology in Kobe, Japan, for example, ophthalmologist Masayo Takahashi is hoping to gain approval soon for the first clinical trial of an iPSC-based treatment, for age-related macular degeneration, in which portions of the ►

► retina begin to die. Takahashi's goal is to replace the diseased parts of the retina with reprogrammed skin cells.

Meanwhile, basic researchers are exploring 'transdifferentiation': a genetic approach that converts one type of cell into a completely different one, skipping reversion to the stem-cell phase entirely. An example is the work of Rudolf Jaenisch and Yosef Buganim at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts. Using a process based on cell culture, the researchers have shown that connective-tissue cells can be transformed into cells that express markers specific to Sertoli cells, which are normally found in the testis³. The results may help researchers to solve the puzzle of male infertility, and may pave the way for techniques for growing cell types that are currently difficult or even impossible to culture.

Buganim says the "specific culture medium used is crucial for the particular fate the cells assume". Transdifferentiated cells, iPSCs or cultured neurons each need their own culture media tailored to the needs of the cells and cell type. The media might have or lack certain growth factors, for example, or create high or low oxygen levels, all of which allow cells to retain their normal characteristics and properties, says Buganim.

More generally, stem-cell researchers in academic or industry labs need media and substrates to maintain and grow their cells, and to coax them down a series of developmental pathways, says Bradley Garcia, who directs technology and business development at Primorigen Biosciences in Madison, Wisconsin, which develops and sells such products. Media tuned to cells' requirements can also keep differentiated stem cells, whether liver or heart cells or neurons, in culture for days, months or even more than a year.

Cells can be unpredictable, growing more readily in one medium than another for no apparent reason. And stem cells, according to Scott Monsma, senior director of research and development at Primorigen, are "balanced on a razor-edge", and will differentiate in response, for example, to rough handling, overcrowding and stress. These factors make stem-cell-media development challenging, but at the same time, the medical potential of stem cells raises demand for such media, companies say.

LESS CAN BE MORE

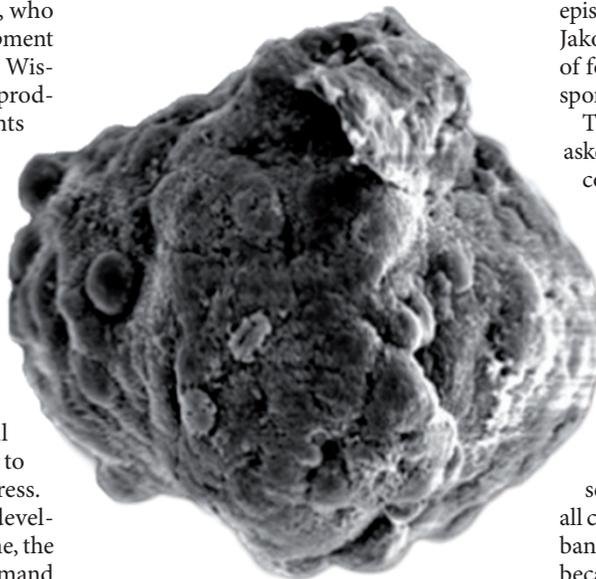
Some scientists use feeder-based systems to get their stem cells growing. In these systems, a layer of supporting cells, such as mouse embryonic fibroblasts, supply the medium with growth factors. But these systems can be prone to error, warns Erik Hadley, senior scientist in research and development at Stemcell Technologies, a spin-off from the British



Glauco Souza has made microtissues with *in vivo*-like morphology and protein production.

Columbia Cancer Agency that is based in Vancouver, Canada, and sells stem-cell media. Not only is each batch of feeder cells different, but it is also hard to control the amount and timing of excreted growth factors, making it difficult for researchers to know which ingredients make cells respond in what way.

To combat these issues, Stemcell Technologies sells a feeder-free stem-cell maintenance medium called mTeSR1. A follow-on product, TeSR2, is completely free of animal proteins,



An artistically enhanced scanning electron micrograph of a liver-cancer-cell spheroid.

and another, TeSR-E8, which was released in January, contains a set of eight components with a formulation based on work by James Thomson, a stem-cell researcher at the University of Wisconsin–Madison. The media are sold by Stemcell Technologies under patent licences from the university. Life Technologies in Carlsbad, California, also sells a version,

called Essential 8 Medium.

Defining exactly what has gone into a culture medium takes the field beyond alchemy and helps scientists to reproduce findings from colleagues, as well as to approach clinical applications, says Mikhail Kolonin, a stem-cell researcher at the University of Texas Health Science Center in Houston.

NO MORE ANIMAL PHARMA

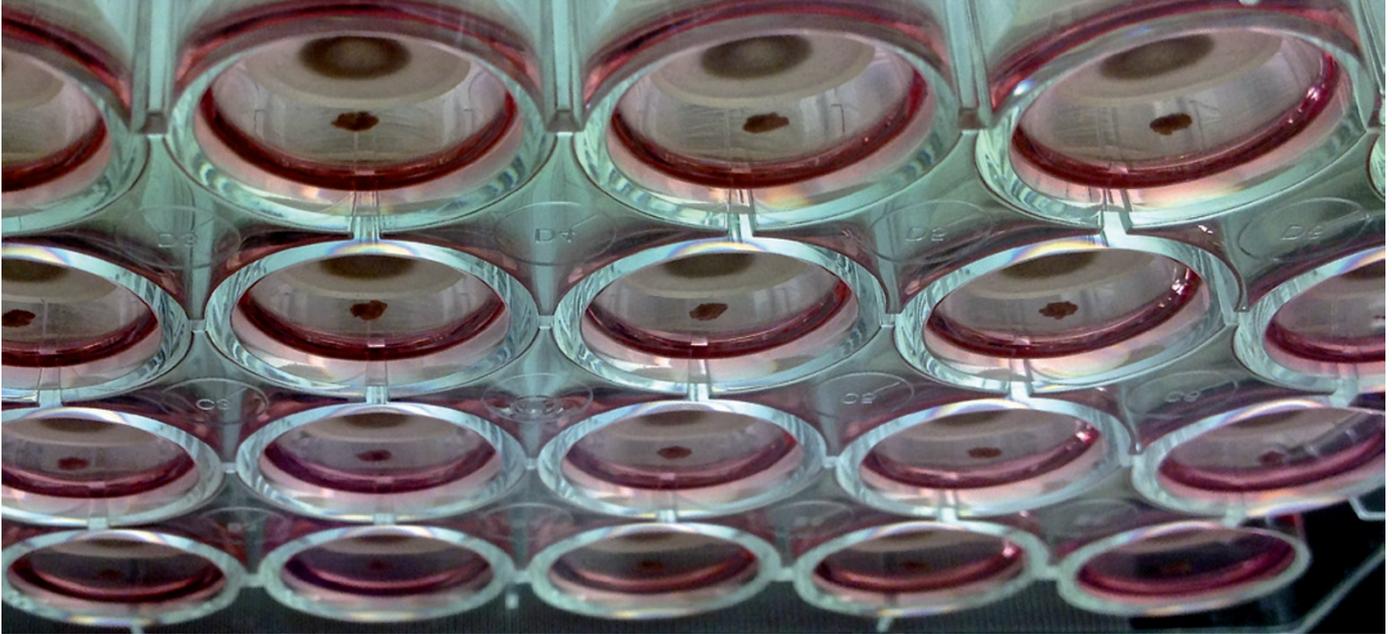
In addition to problems with feeder systems, another stumbling block to reproducibility is that the growth factors, proteins and other nutrients in stem-cell media typically come from fetal bovine serum, which can comprise up to one-fifth of a medium's volume, says Monsma. Each batch of serum — which is part of the blood — comes from a different animal and contains different amounts of components. "The point is, we don't know what's inside," says Kolonin. That is one reason why stem-cell scientists eyeing clinical applications are becoming wary of using products that contain serum.

Another reason is that cells grown in animal products for use in tissue transplantation can "potentially cause an immune response in patients", says Kolonin. Contamination can have even more serious consequences, as the experience with mad cow disease showed, says Nathan Allen, a product marketing manager in the cell-culture and bioprocessing business at Thermo Fisher Scientific, headquartered in Waltham, Massachusetts. In the mad-cow episode, a UK outbreak of variant Creutzfeldt–Jakob disease was caused by contamination of food with the infectious agent of bovine spongiform encephalopath.

The US Food and Drug Administration has asked manufacturers to avoid animal-derived components in therapeutics. This preference affects preclinical research, because ideally, technology choices in the early stages of development should set the pattern for manufacturing further down the road, says Roberta Morris, business director at Thermo Fisher Scientific.

For that reason, new commercial media are increasingly serum-free, says Allen. His company offers a number of serum-free media and defined media free of all components originating from animals. But banishing animal products is not easy, if only because converting media containing serum or with undefined supplements into a more chemically defined version means massively reworking a proprietary recipe, which affects manufacturing.

All of this can make media expensive. Last autumn, Sigma-Aldrich in St Louis, Missouri, launched a stem-cell maintenance medium as part of its Stemline series. The medium is not completely free of animal products, but is composed of defined components and does not contain the types of crude protein ►



Technology by n3D Biosciences brings cells together in a dish using magnetic nanoparticles to levitate them in a magnetic field.

► preparation found in many formulations, such as serum or pituitary extracts, says Dan Allison, principal research scientist at Sigma-Aldrich. It was designed to cater for labs that are working towards industrial applications for stem cells and that will need high volumes of media, says the company.

Monsma says that creating media without animal components, using only chemical compounds and supplements of non-animal origin — such as human serum albumin or recombinant growth factors — means that the proteins must be expressed in human cells or bacteria, then purified and tested. His company and others are setting up capabilities to manufacture such media. For example, Prim-origen is collaborating with several university labs to convert a differentiation medium into one that is animal-component-free.

For stem-cell researchers, shifting away from animal products means abandoning some lab staples, such as mouse feeder cells. Furthermore, some substances traditionally used to coat cell culture dishes are not animal-free. Matrigel — a product that was previously made by Becton, Dickinson of Franklin Lakes, New Jersey, but was sold to Corning Inc. in Corning, New York, last autumn — is a gel used to coat dishes, and is derived from a type of mouse tumour. Researchers at the University of Michigan in Ann Arbor have noted that, although Matrigel has helped scientists to define what iPSCs need, its animal origins and variability are problematic if cells are being cultured as eventual patient therapeutics⁴.

ENGINEERING IN THE MIX

Some scientists will be satisfied only by mixing their own cell culture media. They “tend to know what they are doing and be highly

experienced”, says Masters.

But most, he adds, “are generally not interested in the basics of how to do it properly, just the end product”. They want to be able to buy media off the shelf. Companies have begun catering for scientists who want more defined media. Firms interviewed by *Nature* say that their products are superior to home-brew because they can exert more quality control over the way they source, store, mix and evaluate ingredients, and can manufacture media under controlled conditions.

Engineered media can make a difference. For example, stem cells are deep frozen until their use in the lab, with scientists using a variety of cryopreservation media, including home-brews. But an ongoing challenge in the field is that most cells do not survive the thaw, says Hadley.

Towards the end of last year, Thermo Fisher Scientific began to sell a serum-free, animal-origin-free cryopreservation medium called HyCryo for standard cell lines, and a separate one, HyCryo-STEM, for stem cells. HyCryo-STEM is engineered to improve the recovery rate of stem cells after thawing. Scientists working with neural stem cells can usually recover only 10–20% of cells, and increasing that proportion is not easy with the typical home-brewed freezing media used in labs, says Cindy Neeley, a cell-culture specialist at Thermo Fisher. In tests, the company’s new medium is as good as home-brew, and for neural stem cells the recovery increased to 50–60%, she says.

Improving cell-culture environments also means improving containers.

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Taking an engineering approach, Po Ki Yuen, a bioengineer at Corning, has built a 96-well plate that nurtures growing cells while removing waste — which is toxic — and replenishing media, without an external pump⁵. Not only does the plate require less than the usual amount of daily media exchange, says Yuen — thus minimizing the need for human intervention and lowering the risk of contamination — but it also has fluid movement that is a bit more like that of the body than that of a classic lab vessel.

The idea for the plate, which emerged during a product-development session with two colleagues, he says, is to take advantage of pressure differences between wells that contain different amounts of fluid. Narrow strips of filter paper or a cellulose membrane connect the wells, so that fluid is forced to flow in a controllable way into the adjacent connected well until the liquid heights reach the same level in both. “The flow rate in our perfusion plate can be controlled by liquid height difference between connected wells, and the dimensions and pore size of the strip of cellulose membrane or filter paper,” says Yuen.

The 96-well version is not yet on the market but a 6-well version is, says Brian Douglass, a business-development manager at Corning. Cells in the 6-well version can last for at least 72 hours without media exchange, says Yuen. And, says Douglass, less-frequent media exchange means that “researchers get their weekends back”.

SPHERICAL THINKING

Scientists and companies are also exploring 3D environments in which to foster tissue-like growth of cell clusters, keeping cells close and in constant communication. In this kind of architecture, stem cells can grow into rounded

M. KOLONIN/UTHS

aggregates called embryoid bodies, which is part of the differentiation process.

This means that cells must not attach to the surface of their container, because if they do, they will grow in a spread-out single layer, says Neeley. Thermo Fisher Scientific has developed a series of dishes and multiple-well plates with a polystyrene surface that offers low-adhesion properties. Scaffolds can be used to shape cell clusters as they grow, but the three-dimensionality collapses once the scaffold is removed, like a tent without its supporting poles. They can also block a researcher's view through a microscope.

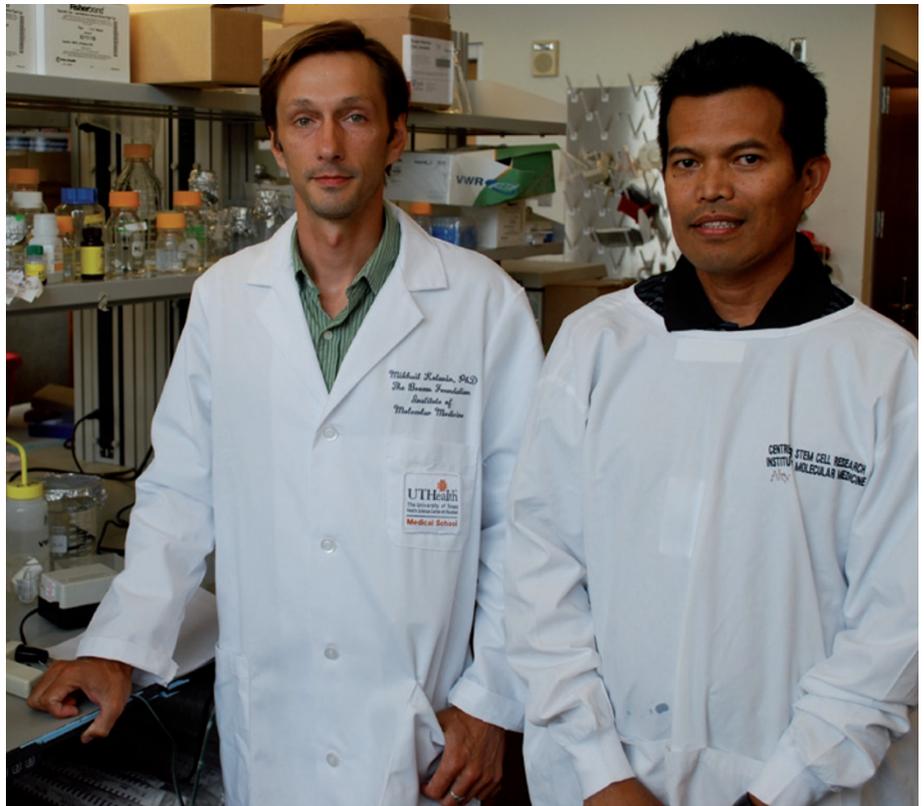
To cater for researchers seeking viable 3D cell-culture options, Thermo Fisher Scientific has developed a culture plate called Nunclon Sphera. "The cells, instead of sticking to the surface, aggregate with themselves and form a three-dimensional sphere in the culture environment," says Neeley. When cultured in this plate, cells grow into spheres that the scientists can transfer from one vessel to another using a pipette, without disrupting the form, she says. Customers are currently beta-testing the product.

Other plate-focused efforts rely on more radical architectural changes. Three-dimensional cell culture dates back more than 100 years, says Ross Harrison, a biologist at Johns Hopkins University in Baltimore, Maryland. He cultured neural tissue in a hanging drop of frog lymph and was able to observe live nerve cells sprouting axons, the long extensions through which neurons send messages to other neurons.

Now, a Swiss company, InSphero in Schlieren, is using the hanging-drop technique as the basis for a multiple-well plate made of the conventional polystyrene but with redesigned wells. After building a prototype, the Swiss Federal Institute of Technology (ETH) in Zurich set up the fledgling firm in the institute's technology park, says Jens Kelm, a biotechnologist formerly at the University of Zurich who founded InSphero four years ago along with University of Zurich colleague Wolfgang Moritz and ETH engineer Jan Lichtenberg. The firm is just moving into its own facilities.

Unlike typical round-bottomed wells, InSphero's wells have a V-shaped lower part, similar in appearance to a champagne flute. At its very bottom, the well is flat. In a hanging drop of medium, cells settle and grow as spheroids in a way that enables microscopy, says Kelm.

Getting the cells into the well also meant changing the well openings, which are shaped like a very narrow flower vase so that they fit tightly around a pipette tip. The researchers found that air-tight contact between the well opening and the pipette tip allowed them to deposit near-identical amounts into each well, which is important for making sure that the results are comparable between wells. To arrive



Mikhail Kolonin (left) and Alex Daquinag at the University of Texas study adipose tissue and stem cells.

at the design, says Kelm, "we started experimenting with pipette tips, cut them off and put in drops from the top and looked at how they came out at the bottom".

In 2011, InSphero began a partnership with PerkinElmer in Waltham, Massachusetts, allowing the plates to be incorporated into PerkinElmer's automated screening instruments, which are used by drug companies. What began as a marketing deal has morphed into the companies collaborating on assay development; for example, they create plates that hold spheroids of liver microtissue ready for drug toxicity tests.

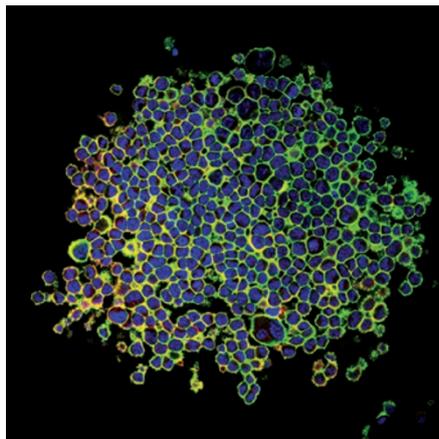
Kelm sees a broad international market for his technology. European laws that prohibit

the use of animals in cosmetics testing have left the industry clamouring for *in vitro* models, such as his microtissues. Drug developers and chemical companies also want cell-based assays to test toxicity. And hanging-drop technology can be used to culture stem cells, an area that could expand as these cells move towards medical applications.

Nadia Benkirane-Jessel, a biologist at the French National Institute of Health and Medical Research (INSERM) in Strasbourg, uses InSphero's technology to investigate ways to shorten recovery times for people undergoing bone-repair procedures and, potentially, bone regeneration. To position bone cells correctly for growth, Benkirane-Jessel seeds cells that have grown into spherical microtissues onto 3D nanofibres developed in her lab for use in mice. She also plans to use InSphero's technology for a product developed by her spin-off company, Artios Nanomed in Strasbourg, in the field of bone and cartilage regeneration.

CELL LEVITATION

Another academic spin-off that is developing 3D cell culture is n3D Biosciences in Houston. As chief scientific officer and company co-founder Glauco Souza explains, the technology seeds tissue by levitating cells and bringing them together⁶. The first step is to decorate cells with NanoShuttle, the company's magnetic nanoparticle assembly of gold and iron oxide crosslinked with polylysine, he says. Next, the cell culture dish is exposed to a magnetic field. "When the magnetic ►



Microtissues such as these colorectal-cancer cells can be grown in spheres without scaffolds.

INSPHERO



Stem cells are often kept in liquid nitrogen at -196°C , but the recovery rate is low.

► field is applied, it brings the cells together while levitating them,” he says.

What keeps cells growing, Souza explains, is the cell–cell interaction that the levitation process promotes, which is more like the body’s environment than conventional cell culture. The technology also makes media exchange easier, because a magnet can hold the tissue in place, he says.

Souza, a physical chemist formerly at the MD Anderson Cancer Center in Houston, says that research with this technology at the company and in collaborating academic labs shows that the resultant microtissues have *in vivo*-like morphology and protein production, enabling them to be used in *in vitro* drug-testing models. n3D Biosciences has customers in university labs and pharmaceutical companies, and is focusing on high-throughput toxicity testing and drug development.

OVERNIGHT SUCCESS

Kolonin uses the technology to study fat tissue, and also considers it a possible environment for growing stem cells into organs. Recreating an organ in a dish requires all the organ’s cell types to be present and to make connections. In a flat dish, however, one cell type usually takes over because it happens to respond best to the media or to the plastic, and other cells are quickly lost, he says. That situation is different with the n3D technology. “You plate cells out, throw particles at them ... overnight, and put them into the magnetic field, and the next day you already have the spheroids,” he says. The spheroids include all the cell types. “It literally takes one day.”

Magnetic levitation has been a good way to model adipose tissue, Kolonin adds, and to culture stem cells while retaining their ability to differentiate⁷.

The magnetic particles may cause adverse consequences for the cells that contain them, but, these are a minority of the cells in culture. The microtissues stay together and the cells tend to spit out the particles, which then remain in the matrix outside the cells.

“There has been a boom of late in 3D formats, and I think the field is rapidly adopting and critically evaluating these technologies,” says Jeffrey Morgan, a bioengineer at Brown University in Providence, Rhode Island. He

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thinks that when cells contact, interact with and communicate with other cells rather than with artificial scaffolds, the cell culture more closely replicates the *in vivo* environment, especially that in solid organs such as the heart and liver, where cell density is high.

Morgan invented what he calls the 3D Petri dish, and in 2009 he founded a company: Microtissues, based in Providence. In a deal that went through last year, Sigma-Aldrich is distributing the dish. Morgan’s customers are academic biomedical researchers, pharmaceutical firms doing toxicity testing and cell-therapy companies exploring how to prepare cell clusters for possible transplantation.

The 3D Petri dish came about when, to guide the growth of tissue-like multicellular spheroids, Morgan and his graduate student Anthony Napolitano began making moulds in the lab. They wanted a material that was non-adhesive for cells, which is the “direct opposite” of the classic plastic Petri dish, says Morgan. At the same time, the researchers needed a

material that would not interfere with the small cell–cell adhesive forces that drive cell clustering. The material they chose was agarose, which forms a commonly used hydrogel and is made of 98% water, which is why Morgan says that his approach is “like sculpting water”.

A user casts molten agarose in the micro-moulds, allows it to gel, then removes the micro-moulded agarose and places it in a standard multiple-well dish. Cell media and cells are pipetted into the dish, and cells then settle by gravity into each of the micro-wells and self-assemble into a multicellular spheroid at the bottom of each moulded well. The micro-moulds can be autoclaved and reused to cast more gels.

The mould makes spheroids that are uniform in shape, says Morgan. Their size can be varied with the number of cells that are seeded. The cells are easier to harvest than in scaffold-based methods. They spill out when the gel is inverted, allowing further tests.

HOPES FLYING HIGH

Although stem-cell research and advances in cell culture are quickly advancing, viable cell therapies are years away from the market, says Chuck Oehler, chief executive of Primorigen. But companies such as his regularly get calls from people seeking stem-cell-based cures. Neither are stem-cell scientists immune to hope.

A researcher who did not wish to be identified is diabetic, and has been dependent on insulin for more than 30 years. A few years ago, he had a transplant of insulin-producing islet cells from cadavers, which allowed him to go for nearly one year without insulin injections and also lessened some of his symptoms, such as numbness in his fingers and toes.

“So the potential seems to be there, if the work we and others are doing to ensure production of cells with adequate, lasting function can be produced,” says the researcher. As a practitioner, he knows the scientific reality. “But given the impact that regenerative medicine can have on my quality of life and on my loved ones, it is easy to understand how those less familiar with the science and industry can be frustrated or impatient with the rate of progress.” ■

Vivien Marx is technology editor for *Nature* and *Nature Methods*.

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