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eBook: 3D cell culture

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A shave new world: hairy skin organoids become a reality

Written By Caitlin Killen

New research has demonstrated it is possible to grow human skin organoids embedded with fat and nerve cells that possess the ability to grow hair follicles.

A new culture technique developed as part of research led by Karl Koehler (Boston Children's Hospital, MA, USA) has resulted in the creation of the most accurate skin organoid model to date. The findings – published in Nature – have implications for wound and burn treatments, as well as potential applications in cosmetics and pharmaceutical testing.

While skin cultures are not a new concept, previous models have lacked vital characteristics necessary for the accurate modeling of normal skin conditions – such as fat and nerve cells, hair follicles and sweat glands.

Previously, in 2018, the team demonstrated that pluripotent stem cells from mice could be utilized to generate skin organoids capable of hair follicle induction.

In this work, the same principles were applied to human induced pluripotent stem cells. A cocktail of growth factors and small molecules were applied to induce the differentiation of the cells.

The first milestone of growth observed was the co-development of the epidermis and dermis, followed by the budding of hair follicles at approximately day 70.

The organoids developed fat and muscle cells; a potentially crucial step highlighted by the study's first author Jiyoon Lee. "The fat is an unsung hero of the skin and recent studies suggest it plays a critical role in wound healing," explained Lee.

Sensory neurons and Schwann cells were also produced, with these nerve cells targeting Merkel cells, mimicking the human touch response.

The team then wanted to see if the organoids could be transplanted onto a live host. To get to the root of this problem they cultured organoids for over 4 months and grafted them onto nude murine models.

When the transplanted skin was compared to human skin samples, several features unique to human skin were observed, including the development of elaborate sebum glands and rete ridges, which are responsible for the anchoring of the epidermis into skin membranes.

Not wanting to split hairs, Koehler warned that we shouldn't view this development as a potential cure for baldness, he explained: "Immune rejection is a major hurdle and generating follicles tailored to an individual will be incredibly costly and take a year or more."

This study serves as a proof of concept and it is too early to make 'bald' claims; however, the authors hope the technology could be used in the future to create cultured skin capable of

Sources:

1. Lee J, Rabbani CC, Gao H ***et al.*** Hair-bearing human skin generated entirely from pluripotent stem cells. ***Nature*** doi:10.1038/s41586-020-2352-3 (2020) [Epub ahead of print];
2. https://www.eurekalert.org/pub_releases/2020-06/bch-chh060520.php

Modeling a heart attack: researchers develop human cardiac organoids

Written By Katie Gordon

Researchers have developed human cardiac organoids in order to model myocardial infarction and drug cardiotoxicity.

A team of biomedical researchers from the Medical University of South Carolina and Clemson University (both SC, USA) have succeeded in developing human cardiac organoids, less than 1mm in diameter, that can replicate the physiological conditions that occur during a heart attack.

"We were essentially able to take that 3D complex nature of a heart attack and then downsize it into a microtissue model," remarked Dylan Richards (Clemson University), a co-author on the study which was published recently in Nature Biomedical Engineering.

In the USA, a heart attack occurs every 40 seconds. Obtaining a sample immediately after a heart attack occurs is very difficult, so the development of an accurate model, such as this one, is vital for helping researchers to fill in the gaps and help to reduce the burden of this condition.

To achieve this, the team, led by Ying Mei who holds a position at both institutions, used induced pluripotent stem cells that divide and mature into different types of heart cells which interact to allow the organoid to act as the heart would.

This technique overcame the limitations associated with the traditional methods, such as using cells in a dish or animal models, which are both unable to model what happens in the human body to a high degree of accuracy.

"The hearts of rats and mice beat five to 10 times faster than those of humans," Richards explained. "How those mechanisms work physically – the electrophysiology and the pumping action – is just different because of the scale."

As well as modeling what happens during a heart attack, the cardiac organoids allow researchers to test the effectiveness of specific heart attack drugs, in addition to monitoring the safety and toxicology of heart drugs in patients with diseased hearts as well as healthy ones.

In the near future, the team intend to incorporate immune cells into the model in order to develop a deeper understanding of the role they play in the restructuring of heart tissue after damage from a heart attack. They also intend to investigate how a patient's genetics impacts their recovery by recreating organoids from patients with different outcomes.

"We are not the first ones to recapitulate the cellular or even the tissue-level response. I would argue, however, that we are the first ones to recapitulate the organ-level response," concluded Mei.

Source

1. Richards DJ, Li Y, Kerr CM ***et al.*** Human cardiac organoids for the modelling of myocardial infarction and drug cardiotoxicity. ***Nat. Biomed. Eng.*** 4, 446-462 (2020).

David Spector: utilizing organoids to study breast cancer

Written By Tristan Free

As part of our Spotlight on 3D cell cultures, we spoke to David Spector, Professor at Cold Spring Harbor Laboratory (CSHL; NY, USA) where he is also the Director of Research. David uses 3D cell cultures as a vital tool in his research, using them to identify long-noncoding RNAs of interest in breast cancer and then to screen potential therapeutics for the disease.

What are you currently working on in your lab?

A major focus in my lab is on long noncoding RNAs, which are a very interesting group of molecules. If we look at the human genome, for example, about 34% of annotated genes are protein-coding genes. And yet about 80% of the genome is transcribed. So, we are very interested in this 'nonprotein coding' region of the genome. And among the nonprotein coding region, the largest class of genes are long noncoding RNA genes. These represent about 27% of annotated genes in the human genome. We're interested both in the basic biology of long noncoding RNAs, which are thought to be regulatory molecules, and in trying to identify long noncoding RNAs that play a critical role in breast cancer. Our real motivation is trying to identify new therapeutic targets that can impact disease progression and then identify drugs that can be used to reduce the level of those long noncoding RNAs and help patients.

How do you use 3D cell models in your work?

We use them in multiple steps. CSHL has a fantastic strategic alliance with a major healthcare system in the New York metropolitan area called Northwell Health (NY, USA) and I have a protocol in place with several surgeons at Northwell so that I am able to receive fresh patient tumor samples within about two hours of resection from the patient. These are delivered directly to my laboratory. We then take those samples and we develop them into 3D cell cultures, or 'organoids'. The goal here is twofold; one is to identify long noncoding RNAs that are upregulated in those tumor samples, versus normal samples from the same patient and then a second goal is to use the organoid lines that we're developing for drug screening approaches.

What challenges do you face in those in the development of those tumor organoids from the tumor samples?

It all comes down to the complexity of disease. Breast Cancer is a very complex disease and we find that organoids we try to make from patients vary greatly in their growth properties, and the ability to passage them over multiple passages in 3D cultures. We do quite well, putting them into the initial culture our take rate is quite high. However, we have great variability once we start passaging them. Some do extremely well, some not so well. It's not simply related to a tumor grade or a tumor subtype. So, we're trying to understand what other factors might be playing a role in the ability to keep these lines going for a considerable amount of time.

Once you have that finished product of the tumor organoids, what are some of the key techniques that you utilize with these organoids?

The first thing that we do is fully characterize them. We perform RNA-sequencing analysis, copy number variation, we have a mutation panel that we assess. Then we do some immunohistochemistry looking at Ki67 for example which is a marker of cell proliferation.

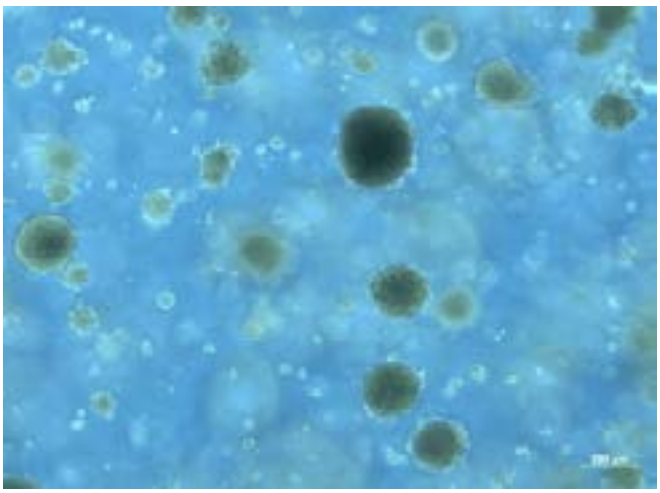
Essentially, we're trying to gain as much knowledge as we can from each of these organoids lines so that we can fully understand their genomic features and output and then how that might impact disease progression.

Do you have any tips for best practice or pitfalls that fellow researchers should look out for when conducting those techniques with 3D cell cultures like organoids?

Right, I think the critical thing that one needs to keep in mind is standardization. This is because the media that is used – and we're using a medium that was developed at Hans Clevers' lab (Hubrecht Institute, Utrecht, The Netherlands) – is extremely complex. So we have one person in the lab that focuses on making up the medium that everyone else in the lab is using and that way we can compare data from multiple individuals in the lab. This means we don't have to worry about slight variability in media contents for various experiments.

When multiple labs are developing organoids, certainly different labs are using different types of media, and one has to always keep in mind that those differences in media composition will certainly lead to changes in gene expression profile. And that will also lead to potential changes in the phenotype. I think standardization in the field is a major area that needs to be paid more attention.

So, are there any ongoing attempts or programs to introduce this standardization into the field of 3D



Patient-derived breast tumor organoids. Sonam Bhatia and Suzanne Russo, Spector lab.

cell culture?

There are a lot of discussions. You know, people are looking at various published protocols and deciding which ones they will use for their particular experiments. It's not like 2D culture where everybody uses DMEM plus 10% FBS for many different culture types. It's much more complex. You have to be concerned about the growth factors that you're adding and the activity of those growth factors when you purchase them from different companies. You can't be 100% sure that the activity is going to be identical.

These are some of the things that need to get worked out and I know a lot of labs are interested in getting this standardized. I'm sure there are also a number of companies that are trying to play a role in helping to standardize media conditions, etc. I am confident in the long term, just like we buy DMEM, there will be commercial products that will be worked out that are standard. That would be a great thing to see.

With the complexity of the media evolved, does that also bring challenges with contamination?

Fortunately, we don't have any contamination issues in our lab, but it is something that we are quite neurotic about. We do all of our culture work in laminar flow hoods, the incubators that we use are only for organoids, we have a separate room where we do our organoid only culturing; we don't allow anyone to bring, for example, mouse cells or HeLa cells into that room. We are just very careful and we try to control things as best we can.

When utilizing tumor organoids in your research, how do they compare to animal models in your studies? What are the pros and cons of the two?

No one system is the one and only. You can learn things from many different systems and I think that's the best approach. You learn a lot from 2D culture but you don't learn everything, and it's similar with organoids. I don't think that organoids are going to totally replace mouse models. They certainly add a significant capability. But we still do mouse experiments in my lab and we try to glean what information we can from the mouse or human organoid models and in some cases even some 2D culture. We bring all that information together to develop our research goals.

The big advantage of organoids is that these are true patient samples, which are extremely valuable. In terms of our studies and in terms of long-term costs, I think organoids have a big advantage over mouse models. Keeping huge mouse colonies going is more expensive and labor intensive.

Organoid models are more scalable as well because we can have 50 different patient samples, which we have a biobank of. We can ask our question many times and under different experimental conditions using the samples from this biobank. We couldn't do that in a large number of different mouse models. It would be impractical.

Can you identify any discoveries that tumor organoids have facilitated that you wouldn't have been able to uncover had you just been using animal models?

With 3D organoids, we have the ability to identify long noncoding RNAs that are upregulated in breast tumors versus normal mammary epithelial cells from the same patient, as they are derived from patient samples. So we have, in a sense, the perfect control. We're not just pulling out some normal cell line and comparing it to our tumor cell line. We're taking material from the same patient and doing comparative studies. I think that's extremely valuable.

It has allowed us to identify some long noncoding RNAs that are upregulated in tumors. And we're currently working on them to try to understand their molecular function.

Do you have any idea, as of yet, what the molecular function of those identified, long noncoding RNAs could be?

So, we're very early in our studies there but we have an interesting story. In one case, we initially start working on a long noncoding RNA, in our mouse models. Then we identified the human ortholog, and then pursued it in our patients' tumor organoids. We then did a comparator to see how well-conserved function is for this long noncoding RNA between mouse and human.

A really cool experiment that we did was to knock out the mouse gene for this particular long noncoding RNA and then after identifying the human ortholog – identified by synteny, not by sequence identity because long noncoding RNAs tend to not necessarily be conserved by sequence but more by synteny – we cloned the human ortholog. We then expressed it in our mouse knockout cells and showed that we could rescue the phenotype. Thereby, clearly demonstrating that it was in fact, the human ortholog. We are now assessing its function in our human patient-derived breast tumor organoids

What do you most enjoy about working with organoids?

The fact that we're working on samples that are as representative as we can get to evaluating a patient's tumor, and are then able to manipulate that sample in ways that might provide an insight that could potentially help the patient, is really exciting. The ultimate goal here is to be able to take a tumor sample from a patient, put it into 3D culture and

then treat it with a battery of different drug combinations and identify in a patient-specific manner what might be the best drug combination or drug for that particular patient.

If there was one aspect of working with organoid models that you could ask for to be improved, what would it be?

Right now, we need better methods for making knockouts and for tagging various molecules in the organoids. That would be a huge help for our particular work. Also, as I said earlier on, even better optimizations of the growth medium. I think there's still work that could be done there.

Is this an area that CRISPR could begin to assist in as it develops or are there other techniques that you think you would employ?

I think getting the CRISPR methods to work efficiently in organoids would be a huge plus. We're currently taking a different approach. Just to get around the fact that methodology is not yet optimized in organoids. We're using antisense molecules that can knock down particular RNAs. We find in many of our organoid lines that we can achieve about a 95% knockdown using antisense oligonucleotides.

We have a very strong collaboration with a pharmaceutical company (Ionis Pharmaceuticals, CA, USA) that works with us to design these molecules that we can use in organoid lines to assess the impact of knockouts on these systems.

If you had any advice for early career researchers, what would that advice be?

Come up with important innovative questions and innovative approaches to address those questions. Use a wide array of approaches to address your question. Think about the potential impact of what your work will have on your field,

Where would you predict the field of organoid and 3D cell cultures could progress to in the next 5 years.

In the next five years, I think we'll get closer to moving these systems towards being able to impact clinical decisions. I mean we're already starting to move in that direction. And so I would expect that would be a very significant and impactful development.

At CSHL, we have established an organoid facility. The hope is that will strengthen our clinical collaborations. We plan to have part of that facility, set up as a CLIA certified lab that would have the ability to provide insights back to clinicians.

Sylvia Boj on gene editing in organoids and their use in drug discovery

In this interview we catch up with Sylvia Boj, who takes us through a recent paper that used patient-derived organoids as a test case for a new CRISPR technique and talks about the impact this technology could have in the development of organoids for regenerative medicine. We also discuss future developments in organoids, and their involvement in drug discovery and cancer research.

Sylvia Boj is the Chief Research Officer at Hubrecht Organoid Technology (HUB; Utrecht, The Netherlands). Her primary responsibility is to lead the HUB research team and to coordinate all the research activities that are taking place in the organization.

Can you tell us about the updated gene editing technique developed from CRISPR technology that you recently applied in cystic fibrosis organoid models?

In this publication our academic collaborators from the Beekman group at University Medical Center Utrecht and the Clevers group at the Hubrecht Institute (both Utrecht, The Netherlands), who are also the main authors of the manuscript, applied a recently developed Cas9 fusion protein. This new generation of Cas9 fusion proteins can introduce changes into genomic DNA, while circumventing some of the side effects that have been observed by initially developed CRISPR–Cas9 proteins, which were more prone to introduce some deleterious off-target double strand breaks.

The idea was to evaluate the activity of two new Cas9 base editors (SP Cas9 and XSP Cas9) in a very clinically relevant model for cystic fibrosis, intestinal organoids derived from cystic fibrosis patients, with a very easy readout, the forskolin induced swelling (FIS) assay, in which restoration of CFTR function can be easily measured. So, the cystic fibrosis organoid models were used as proof-of-concept for the new CRISPR technique.

Could these new Cas9 proteins help alter aspects of cystic fibrosis or other organoids that you were previously unable to manipulate?

In the context of cystic fibrosis and organoid technology, it is slightly more difficult to reflect on how this new generation of Cas9 base editors can make an impact, but they definitely have an incredible impact in applications for regenerative medicine.

Together with the intestinal model, liver organoids, derived from human biopsies, have shown their potential to engraft and regenerate tissue. We can, therefore, foresee new Cas9-editing based technologies repairing mutations in metabolic genes in liver organoids generated from patients with such metabolic diseases. Repaired organoids could then be transplanted back to the patient to replace the mutant live cells with the repaired ones. This clearly requires a high level of accuracy to ensure existing mutations are fixed without inducing new, off-target mutations in the organoids that are intended to be used for transplant tissue.

What were some of the most exciting discoveries for you from that study?

I think it is very exciting that we have proven in a relevant clinical model that this new generation of Cas9 proteins can repair a gene and do not introduce off-target effects in the genome. The possibility that with this approach could open a window for the genetic-based treatment of certain cystic fibrosis patients. We provided very strong data that pushes forward this approach in terms of improving the treatment of patients with cystic fibrosis, for whom the available drugs cannot restore CFTR function.

What areas you working on in organoid development or organoid application that you find particularly exciting?

The research of organoids in cystic fibrosis is our hallmark and it is the model where we have been able to cover the entire circle in terms of drug development and proving the clinical correlation between organoid models and patient response. This has helped cystic fibrosis organoid models to become an essential in-vitro model for preclinical phases. It is very exciting for us to see that any current drug development in cystic fibrosis is taking place using our organoid models. Furthermore, because of their predictive value, we have been able to include patients in clinical trials where we could identify the optimal treatment for the patients.

We are now working at HUB to translate this success into the field of oncology; this means that we are validating the predictive value of organoids for different types of cancer. We are also using organoids in drug discovery and drug development to find better treatment using a more patient relevant model.

What methods are most effective for using organoids for drug discovery? What challenges do you encounter when using organoids for drug discovery?

For drug development, the most usual readout that we perform in drug-screenings is cell viability. We expose the organoids to a certain drug at certain concentrations and then evaluate the impact of the drug on the viability of the organoids.

A particularly challenging aspect that we encountered while using Cas9 technology in cystic fibrosis organoids was the need to target the stem cell – in this case the stem cells of the intestinal organoid from the cystic fibrosis patients – to generate the clone that has repaired the mutation.

The challenge in oncology research is that to make the tumor organoid model truly representative, you need to mimic the heterogeneity of the tumor and this heterogeneity needs to be preserved. When establishing tumor organoids, we preserve multiple different clones to ensure this heterogeneity. However, this Cas9 application requires a single-cell step in order to efficiently introduce Cas9 into the cells. Due to this single-cell step, you lose the heterogeneity from the clones that are generated after it.

What technologies do you employ to carry out that drug screening? Do you have any tips for best practice when using those technologies?

One of our missions is to translate this organoid technology to industry and clinics and to make it as broad as possible, because we believe it's a very relevant model for drug development. We put a lot of effort into standardizing our protocols and making them very robust. In terms of drug screens, we developed them at a high-throughput level. We dispense organoids with multidrop, and we can plate 40–50 384-well plates to perform drug screens in organoids. We've incorporated this in the use of liquid handlers and also imaging platforms.

Any high content screening microscope systems can also be used to image organoids and do image analysis. Cell viability is still the main readout for experiments in the oncology area, but we are looking for imaging readouts as we do with the swelling assay. The forskolin-induced swelling assay is also presented in the manuscript mentioned previously, is the readout we do for measuring CFTR restoration.

Where do you see the capabilities of your organoid models in 5 years' time? Do you think they will become even more representative or do you see them being employed in new areas?

In 5 years, personalized medicine with organoid technology will be a reality. We will be able to assess what drug a cystic fibrosis patient should take using an organoid assay – our team is working on that now. Currently, we are already running organoid based clinical trials for cystic fibrosis patients. In parallel, we are working to move this application of organoids for personalized medicine and patient stratification for clinical trials in oncology for different types of tumors.

Can you tell me more about how you would employ organoids for cystic fibrosis patients?

We are now working on assay standardization and validation to get approval from the right entities (US FDA, EMA) to implement an HUB organoid-based test for a cystic fibrosis diagnostic, as we have already proven the predictive value of organoids in retrospective clinical trials. An organoid generated from a cystic fibrosis patient can predict if a patient will benefit from a treatment.

In individual cases in the Netherlands, for patients who's lives are at risk and have no other alternative, their organoids have been generated, and if, in a FIS assay, the organoids have responded to a treatment, these patients have been given access to the tested treatment.

Is there anything else you're working on at the moment that you're particularly excited about?

We also have singular patient examples and corroboration from other research studies about organoid predictive value in oncology. For example, for colorectal cancer, organoids can predict patient response to treatment. We are validating this data by analyzing large numbers of patients and comparing patient and organoid data.

Apart of all the efforts for proving organoid clinical validation; the validation of the positive predictive value of organoids in oncology, we are also developing what we could call the 2.0 organoid model. We are combining organoids with other relevant cell types, such as immune cells or microbes to develop organoid models that better represent the pathophysiology of more complex diseases, such as inflammatory diseases, which will allow the testing of the efficiency of drugs in a patient-relevant model.

We are also hugely aware of advances in immune-oncology right now, and the relevance of combining tumor organoids with different cell types like T-cells or macrophages. So, we are developing more complex assays where we can assess immuno-oncology drugs in a relevant clinical model.

Q&A: 3D cell cultures, from development to drug discovery

Kalpana Barnes (Sartorius, London, UK), Robert Vries (Hubrecht Organoid Technology, Utrecht, The Netherlands) and Marta Shahbazi (MRC Laboratory of Molecular Biology, Cambridge, UK) answer your questions following on from the successful panel discussion "3D cell cultures, from development to drug discovery." Don't worry if you missed it, you can catch up on demand now & make sure you check out the rest of our spotlight on 3D cell culture here.

Which types of scaffold do you think are best for the purposes of drug discovery?

Kalpana: Scaffolds that are relatively easy to prepare and handle, compatible with automation and don't interfere with your choice of analytical technique would all be considerations. A good supply chain with associated product quality control would also be desirable. Synthetic scaffolds that offer more precise control of constituents may also be beneficial and preferred over naturally derived scaffolds.

How do we maintain the vasculature in the spheroids or organoids?

Kalpana: There are many advantages to using either spheroid or organoid cultures that we've discussed today. However, one disadvantage is that neither incorporate a vasculature so this a driver for next-generation 3D culture models.

How is it possible to avoid necrosis when the spheroids are developed over 400 microns?

Kalpana: Mass transport limitations mean that spheroids of 400 microns or larger in diameter will display gradients to oxygen, nutrients and metabolic waste products. These spheroids will exhibit a common layered structure consisting of a necrotic core, surrounded by a layer of quiescent cells and proliferating cells at the periphery. Creating spheroids of less than 200 microns will avoid the formation of necrotic cores.

Do you use culture media specifically developed for 3D or do you use standard (or non-standard) 2D culture media?

Kalpana: When using cancer cell lines traditionally grown in 2D and creating 3D spheroids using either scaffold-free or scaffold-based techniques, we are using the recommended media for 2D culture. When culturing 3D organoids, we are using specialized media.

Marta: This depends quite a lot on the type of 3D culture. Spheroids, in general, have less strict requirements in terms of media composition. For example, spheroids of mouse embryonic stem cells or human embryonic stem cells can be formed with a simple basal media. However, organoid cultures require more

complex 3D formulations that were empirically developed. Each organoid type has slightly different media requirements.

How can a "single organ" organoid replace animal trials? Or are you planning to connect various organoids, as is being done in organ-on-the-chip devices?

Marta: This is an excellent question. All models have positive and negative aspects, and we need to choose the appropriate model to address our experimental question. I don't think organoids will completely replace animal trials, at least not at the moment. While they are ideally suited to perform drug screening, they cannot be used to assess drug toxicity or side effects. For diseases that involve multiple organs and their crosstalk, organoids would not be very helpful.

Rob: Animal experiments are aimed at studying biology and, in drug development, at testing efficacy and safety in a physiological system. The main issue with the relevance of data from animal studies for humans is that it most often doesn't reflect results in humans. For this reason, scientists try to develop human models.

With the recent advances in iPSC and adult stem cell-derived organoids we have made a major step in the development of human/patient-relevant models. Therefore, we are able to answer many more of our biological, efficacy or safety questions by using a human relevant model system instead of an animal study. This reduces the use of animals and provides better answers. We will need additional developments to completely replace animals, but it is now coming close.

Human cells such as organoids can be grown in many different systems from simple plastic (multi) well plates to complex microfluidic systems set up on 'chips' often referred to as Organ on a Chip. As above the aim of the model is to represent a human. The choice of system depends on the question that needs to be answered. For example, for testing the viability of cells in response to drugs it is preferable to study only the tumor organoid, whereas to study the interaction of endothelium with a tumor the combination is required. This is important because the combination of organs on a chip in itself is not relevant. It is only useful when we are studying the interaction between the organs AND the organ on a chip model mimics the interaction in a physiologically relevant way.

It needs to be considered when developing combination models of organs on a chip, that the connection of organs in a human body is the practical result of evolution: a liver is connected to an intestine by blood vessels etc., to transfer material or communicate. In a laboratory, we have pipettes to transfer molecules and we can simply grow liver organoids and transfer their molecular communication and material or drug metabolites by pipetting to an intestinal organoid, no need to recreate a blood vessel.

Cancer organoid culture will select for a sub-population of the tumor *in vivo.* Can someone please comment on this?

Rob: Data on HUB Organoids (e.g. van de Wetering et al. Cell 2015) shows that a heterogeneous population of cancer cells obtained through resection or biopsy maintains its genetic diversity in culture after long-term passaging. This indicates that this organoid culture method mimics the in vivo conditions such that no in vitro selective pressure is observed. Of course, in vivo, and therefore in vitro, tumor cells are very sensitive to both cell intrinsic and external selective pressure. Therefore it is very important to monitor long term culture of tumor organoids because the population is dynamic and will evolve over time as will the in vivo tumor.

Which types of microscopes are used to observe 3D cell cultures?

Kalpana: 3D cultures can be observed using a range of microscopes and are dependent on what you are interested in observing and resolution. Inverted light microscopes, confocal and high content imagers are some examples.

Marta: We always use confocal microscopy for whole-mount immunofluorescence. Spinning disc confocal works well for time-lapse experiments of small organoids. Bigger organoids would require technologies that allow deeper penetration, for example light-sheet microscopy.

Has anyone conducted single-cell sequencing on patient-derived organoids and compared their similarities and differences in cell populations and microenvironment?

Rob: There are a number of studies that have performed single-cell sequencing on organoids. A recent one by the Clevers lab (Beumer et al. Cell 2020) shows the identification of different populations of enteroendocrine cells in the intestine. A European consortium of the Human Cell Atlas project is studying differences between cells of the intestine and brain. These studies will characterize the cell population and their response to the environment.

I did not hear anything about scaffold-free 3D cell culture. And will be happy to hear more experiences about it.

Kalpana: There are several techniques you can use to create 3D cultures without using scaffolds. Examples include using ultra low adhesion plates which have a round well bottom and ultra-low attachment coating, hanging drop plates as well as rotating bioreactors, micro-patterned plates and magnetic levitation.

Marta: Scaffold-free 3D cell culture is used for example to make embryoid bodies. This is a technology that allows you to generate aggregates of embryonic stem cells that can spontaneously differentiate and generate a collection of differentiated cells. Embryoid body formation just requires a non-adherent plate and the right media composition.

Which 3D cell culture method is more suitable for studying metastasis or invasion?

Kalpana: One method commonly adopted model is of single tumor spheroid invasion into an extracellular matrix such as Matrigel or Collagen. Here a single spheroid is created in a low adhesion plate, followed by direct addition of Matrigel into the same well, which provides a semi-solid matrix for the tumor cells to invade. The advantages of this method include precise control of the spheroid size allowing you to mimic the defined micro-regions of metastatic solid tumours and since the invasion assay is performed in situ there is no need to transfer the spheroid to a second plate which is common with other methodologies. This method is also highly compatible with automated live-cell analysis.

SARTORIUS

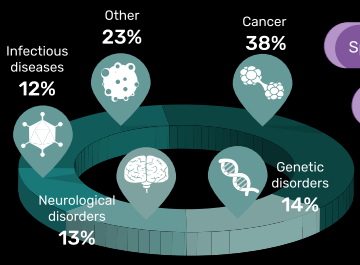
3D cell culture

BioTechniques Spotlight

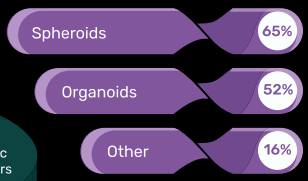
Key trends in 3D cell culture

We asked respondents what words came to mind when they thought about 3D cultures

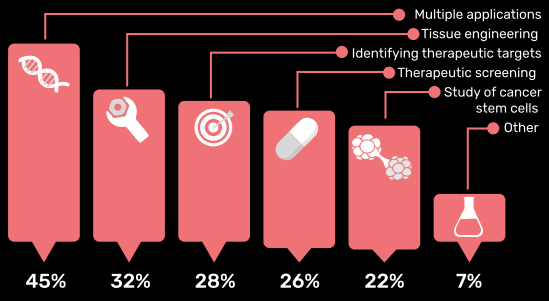
The key disease areas studied using 3D cell cultures



The most popular 3D cell cultures used were spheroids



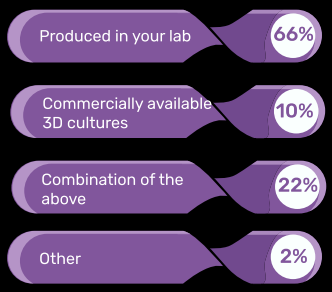
The most common applications of 3D cell cultures



3D cell culture preparation

The majority of respondents produced their own cultures

Where do you source your 3D cultures from?

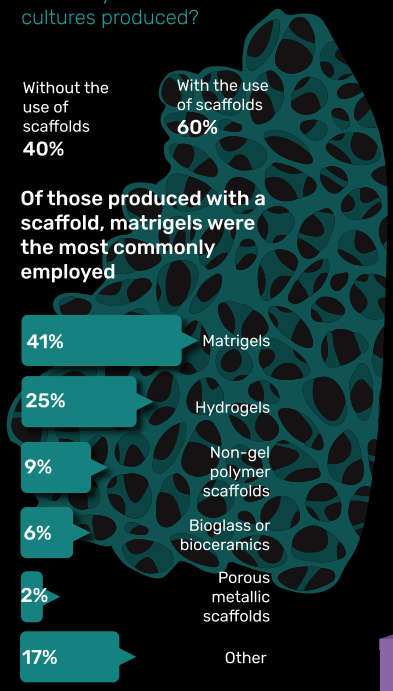


How are your 3D cell cultures produced?

Without the use of scaffolds **40%**

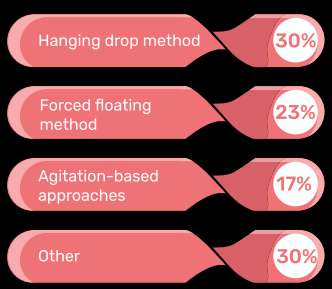
With the use of scaffolds **60%**

Of those produced with a scaffold, matrigels were the most commonly employed



The hanging drop method proved popular among cultures produced with a scaffold-free technique

What scaffold-free technique do you use?

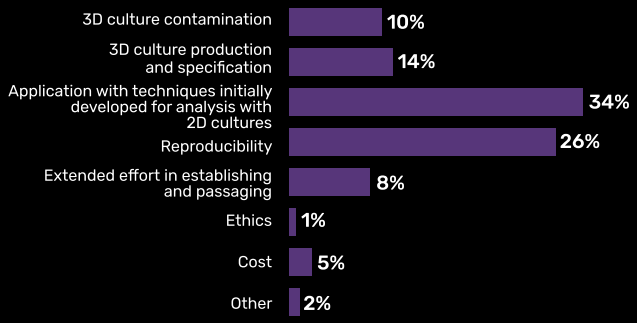


Of the 'other' responses, ultra-low attachment culture plates and clinostat were popular answers

Challenges and contaminations

It seems adapting existing techniques for use with 3D cultures is still of utmost importance as 'application with techniques initially developed for analysis with 2D cultures' was selected as the biggest challenge of working with them

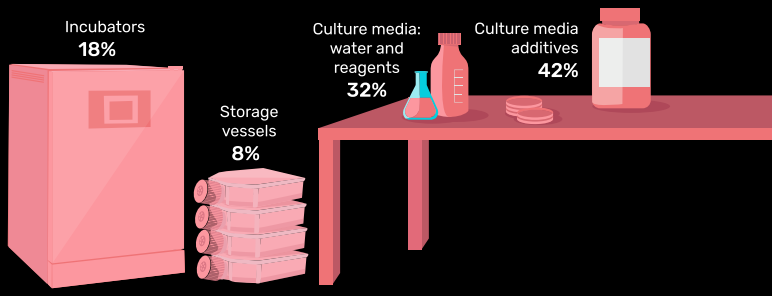
What do you consider the biggest challenges of working with 3D cultures?



Biological contamination was by far the most common type of contamination encountered by users

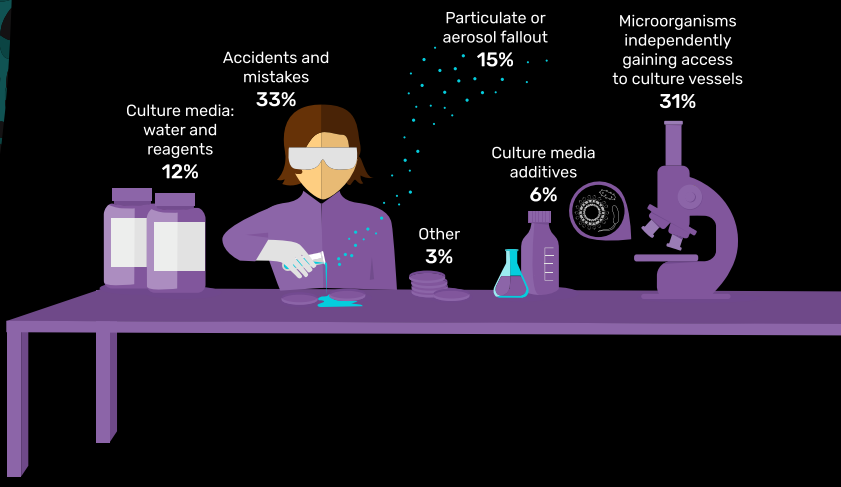
Culture media, particularly additives, were identified as the main cause of chemical contamination

What do you consider the main causes of chemical contamination?



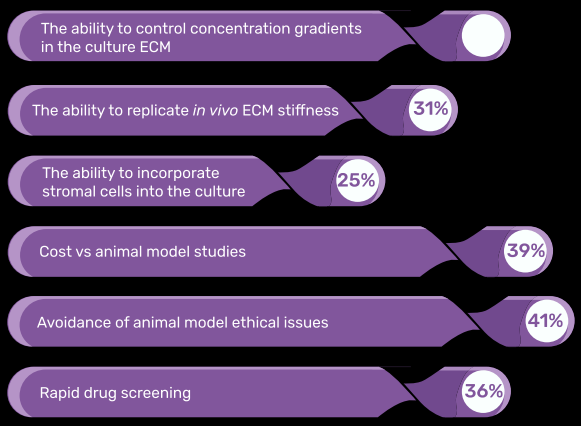
Accidents, mistakes and pesky microorganisms accounted for almost two-thirds of the biological contamination reported by respondents

What do you consider the main causes of biological contamination?

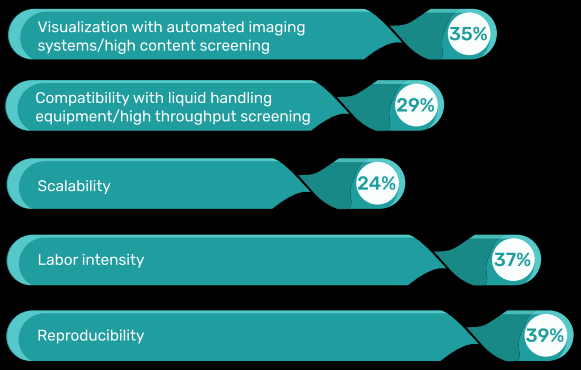


3D cell cultures in drug discovery and beyond

The advantages of using 3D cultures in drug discovery were hotly contested, with 'the avoidance of animal model ethical issues' selected by the highest portion of respondents

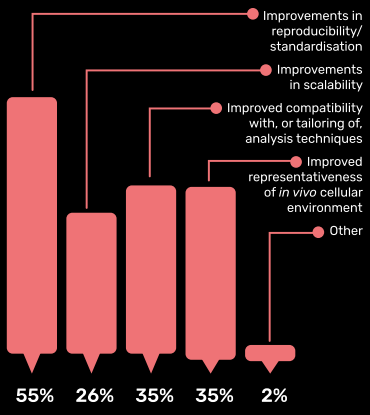


The main challenges were also hotly contended, with reproducibility highlighted by the most respondents as the biggest challenge to implementing 3D cell cultures in drug discovery



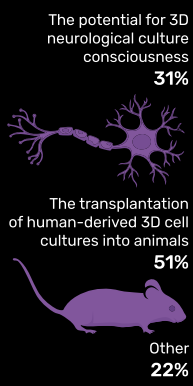
Reproducibility was also picked by the majority of respondents as one of the most important areas in need of improvement with regard to 3D cell cultures

What is the most needed improvement in 3D culture technology?



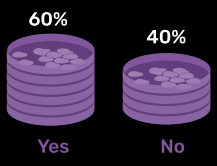
Ethically, the introduction of human-derived cultures into animal models was deemed the most contentious issue

What do you consider the biggest ethical issue in 3D culture research?



About the respondents

60% of respondents work with 3D cell cultures



Which of the following best describes your job title?

Lab director/Chief scientist	10%
Department head	5%
Principal investigator	11%
Technician/Research assistant/Staff scientist	24%
Graduate student / post doctoral fellow	17%
Professor/Instructor/ Lecturer/Assistant/ Associate professor	11%
President/CEO/VP/Owner	6%
Research director/ VP Research/CSO	2%
Product manager	3%
Consultant	5%
Other	6%

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BioTechniques Spotlight

BioTechniques

Seeing the full picture: advances in 3D cell culture microscopy

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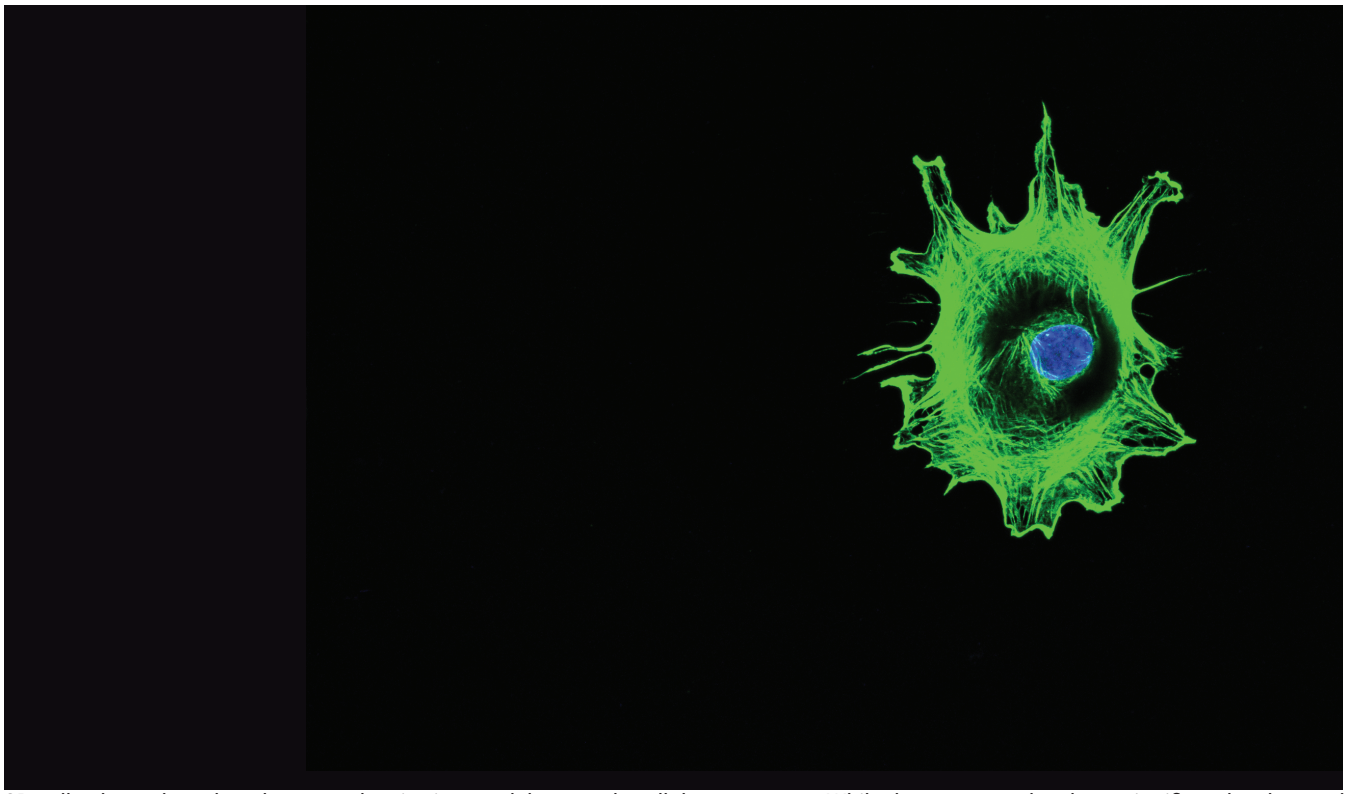
ABSTRACT

As 3D cell models become increasingly intricate, they require more complex tools to analyze them. How are microscopy techniques keeping up with this rapidly evolving field?



2D cell cultures have long been used as *in vitro* models to study cellular responses. While these approaches have significantly advanced our understanding of cell behavior, there are some areas in which they fall short. 2D cell culture is generally insufficient at reflecting the *in vivo* behavior of an organ, and it can be difficult to translate research conducted in 2D cultures from bench to bedside. As a result, animal models are often used; however, differences remain, and thus efforts to translate research to humans can still fail.

Research has seemed to shift towards the rapidly evolving field of 3D cell culture, allowing researchers to recreate organized, miniaturized versions of organs that can be used for various means, such as investigating disease mechanisms and developing new drugs that might be more likely to translate to the clinic than those discovered using other models.

3D cell culture represents a dramatic step forward; not only in the study of tissues and diseases, but also in the fields of drug discovery and analysis. This technology allows researchers to investigate causes of a disease, discover new drugs, test drug sensitivity and identify appropriate, patient-specific treatments. However, challenges still remain, for instance in high-throughput screening, 3D cell-culturing techniques and in maintaining cell viability, as we continue to work towards replicable, biologically accurate organ models.

Despite the dramatic increase in use and plethora of potential, the development of imaging and analytical methods to compliment this technology lags behind. As 3D cell models become more complex, they require more complex tools to analyze them. The majority of analysis techniques used today have originally been developed for 2D cell cultures, hence the transition to 3D is not ideal. This article will focus on the limitations associated with current imaging tools and the steps researchers are taking to develop more sophisticated techniques to tackle these challenges.

Don't put a label on it

Confocal fluorescence microscopy is the current gold standard for studying 3D culture systems. Unfortunately, limitations are still attached to this technique. Hence, there is a real need to develop and introduce a technique capable of studying cells in relevant 3D environments.

Importantly, conventional confocal microscopy is semi-quantitative and requires labeling that can impact cell function and intracellular processes.

Talking to *BioTechniques* at ASCB 2019 (DC, USA, 7–11 December 2019), Bruno da Rocha-Azevedo (UT Southwestern Medical Center, TX, USA) commented on the importance of the right level of labeling: *"If you have too much labeling you're not able to detect single-molecule particles and then you lose the [ability to determine] what is a single molecule. On the other hand, if you label too few, you don't have enough particles to be detected and you cannot actually see the interactions of the receptors."* [1].

Berdeu *et al.* also previously attempted to address the labeling concerns highlighted by da Rocha-Azevedo through the development of a 3D + time lens-free microscopy technique, providing insights into both temporal and spatial aspects of 3D cell cultures. While the correct fluorescence labeling can provide higher resolution and specificity via confocal and light-sheet microscopy, the new technique favored ease of use [2].

There are a number of other imaging techniques that have previously been used to provide detailed visualization of the morphology and spatial distribution of biological structures. An example of this is Raman spectroscopy, which is an inelastic light-scattering technique that can provide label-free biochemical information.

Applying Raman technology, Kallepitis *et al.* sought to answer the need for an endogenous technique that can study cells in highly relevant 3D environments, while providing quantitative biomolecular information of multiple components simultaneously and nondestructively [3]. The team developed a quantitative, label-free Raman imaging approach for visualization of 3D cell morphology and volumetric quantification of biomolecular structures with submicron-size detail. Termed 'quantitative volumetric Raman imaging', the researchers believe this new method will open up new avenues for studying the complexities of cell–material interactions within a plethora of 3D culture systems, revealing new information about cell behavior and function in advanced biomaterials that has previously been difficult or impossible to measure.

There is, however, still a gap when it comes to Raman technology, and concerns arise around the lack of tools for the easy analysis and interpretation of spectral data focusing on biologically relevant information – a point that will be touched on later.

Aspects of da Rocha-Azevedo's concerns do seem to have been answered, but at a cost, be it resolution or specificity. There is still some way to go before dreams of high-level resolution 3D single-molecule imaging of live cells comes to fruition: *"I would love to have a system where I can do single-molecule imaging and tracking in a 3D setting, that I can use to make blood vessels in vitro and image the cells to see how the receptors are behaving [in a situation that is] very close to the* in vivo *setting,"* da Rocha-Azevedo remarked.

Clearing the cobwebs

Optical tissue transparency allows scalable cellular and molecular investigation of complex tissues in 3D. Tissue-clearing approaches have enabled observation of the cellular structures of transparent animal organs. However, adult human organs have proven more difficult due to the build-up of insoluble molecules in older tissues, causing human organs to be too 'stiff' for the animal approach.

To overcome these challenges, a team from Helmholtz Zentrum München (Germany) recently developed a new microscopic imaging approach that has made intact human organs transparent for the first time, raising hope for the development of 3D-printed human organs suitable for transplant [4].

First, the researchers discovered a detergent called CHAPS, which makes centimeters-deep small holes throughout the organ through which solutions can be administered to make the organ transparent. This is possible as CHAPS can aggregate into much smaller micelles compared with standard detergents, such as SDS.

In collaboration with Miltenyi Biotec (Germany), a new laser-scanning microscope – the 'Ultramicroscope Blaze' – was developed that can image large organs.

While rapid progress in tissue-clearing methods has been made both here and elsewhere, this introduces a limitation – how do you analyze these large datasets collected? The team tackled this hurdle through the development of a deep-learning pipeline that can analyze the millions of cells cleared. Combined, this new technology was labeled SHANEL – small micelle-mediated human organ efficient clearing and labeling.

SHANEL is now being used to map some of the major organs of the human body, beginning with the pancreas, heart and kidney (Figure 1). These cellular maps of human organs could be used to engineer large-scale human tissues and organs with emerging 3D-bioprinting technologies, once again highlighting another technological advancement that researchers have developed to overcome existing problems in imaging 3D cell cultures.

3D cell cultures in tumor research

Understanding tumor characteristics by developing an accurate model is the key to understanding the link between various types of cancers. 3D tumor cells grown using 3D cell culture methods have claimed the spotlight in tumor cell biology research because of their innate ability to replicate the *in vivo* environment of a tumor cell *in vitro*.

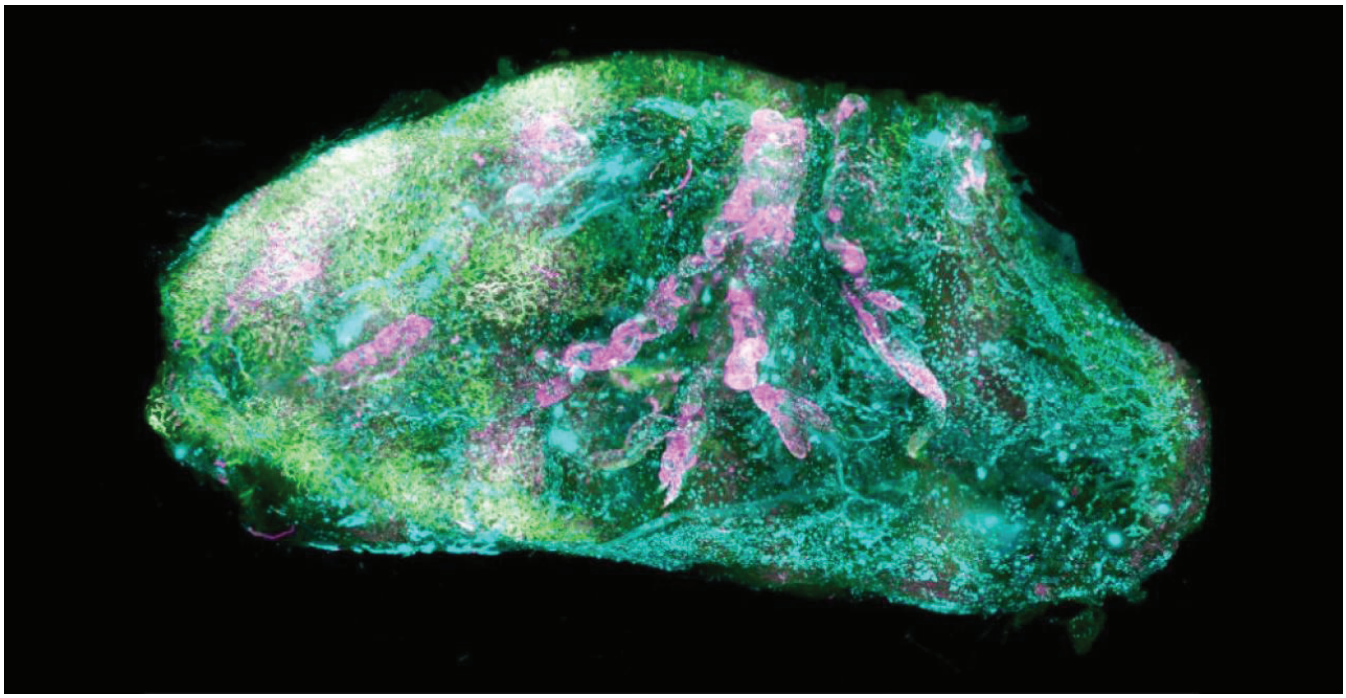


Figure 1. Vascular and glomeruli details of the human kidney.
Reprinted from Helmholtz Zentrum München/Ertürk lab.

Currently the gold standard for conducting brain tumor research is the animal model – as Guohao Dai (Northeastern University, MA, USA) described in an interview with *BioTechniques*: *"You inject the patient's glioblastoma tumor cell into the mouse brain. This is called intracranial transplantation – then you can study how the tumor invades the brain and monitor its response to drugs."* Dai continued to explain that this is a very expensive and lengthy process [5].

Dai *et al.* recently focused on making improvements in glioblastoma modeling and imaging using 3D printing techniques [6]. By 3D modeling *in vitro*, tumor cell growth can be visualized in real time. In this case, the researchers were able to print a 3D vascular channel together with the 3D tumor model, so the tumor model is perfused with vascular channels. This allows researchers to test long term, which is much more reflective of treatment in real life. This is a key benefit of using a 3D model, as even the gold standard animal model has its pitfalls, as Dai explained: *"During the 6 months after you inject the tumor cells into the mouse brain, you cannot tell much about what's happening in the brain and the tumor development until you open the cranial window. Once the cranial window is open you can observe what is happening there, but there is no real-time monitoring of how the tumor interacts with the brain tissue or how they grow."*

As mentioned previously, one of the well-known limitations of 3D models remains with the challenging imaging process. 3D models are commonly very deep, at least a few centimeters. Confocal microscopes can only image less than a millimeter. However, the limitations do not stop there – the reconstruction of 3D images can also take a few hours, leading to sample photobleaching. *"That's a lot of interruption if you do that every day with a few hours of imaging time and it will also disrupt your image,"* explained Dai.

To tackle this imaging problem, the researchers developed a novel imaging technique using a laser to scan the tumor, capturing and tracking all the scattered light from the tissue. A mathematical model that identifies the original location of the scattered photons is then combined to reconstruct the image. It is important to note that this process is conducted rapidly, meaning the sample is out of the incubator for just a few minutes, causing little disruption or damage to the cell.

While this technique does seem to provide an answer for the challenging imaging process of 3D models, this does come at a cost. Increasing both the depth and speed of imaging results in a loss of resolution. Dai and his team were able to visualize that the tumor had overall growth; however, they were unable to image to the cellular level, again answering part of da Rocha-Azevedo's dream for 3D modeling with the ability to monitor in 3D but without the desired resolution.

This combination of novel techniques – using fluorescence imaging to study the bioprinted tumor models – allowing reconstruction of the 3D shape of the tumor and visualization of how it grows in real time is obviously a massive step forward in the realm of imaging 3D cell cultures. There are still large strides to be made, particularly with tumor models. There are a lot of other complicated structures that cannot be incorporated into the 3D tumor model. Within an *in vivo* brain tumor there would be an abundance of other complicated structures such as neurons and microglia, the latter being shown to play a vital role in glioblastoma remission and immune response [7]. Incorporating these components into the model will prove very significant. Moreover, in terms of chemical composition, the hydrogels currently used are still far from actual brain tissue.

Despite it being much easier to form vascular channels accurately with 3D bioprinting, as described in Dai *et al.*'s recent publication, a challenge still remains in how to recreate the extremely tight blood–brain barrier. *"That's a very big challenge that would have a very significant impact in improving the tumor model,"* concluded Dai.

Future perspective

The future significance of 3D cell cultures will not only depend on the advancement of microscopy technology, but also the development of tools for both analysis and interpretation of large spectral data. The research highlighted in this short piece represents just a fraction of the technological strides made in this field. Limitations in current methods are slowly being answered, albeit with some caveats.

3D cell culture techniques provide methods that are key to advancing research in many fields. As the imaging of these 3D cell cultures improve, consequently tumor models, cancer treatment therapies and disease-testing methodologies will follow suit.

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Benchmarks

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3D-printable cell crowding device enables imaging of live cells in compression

Liam P Dow[1], Aimal H Khankhel[1], John Abram[2] & Megan T Valentine*,[2]

ABSTRACT

We designed and fabricated, using low-cost 3D printing technologies, a device that enables direct control of cell density in epithelial monolayers. The device operates by varying the tension of a silicone substrate upon which the cells are adhered. Multiple devices can be manufactured easily and placed in any standard incubator. This allows long-term culturing of cells on pretensioned substrates until the user decreases the tension, thereby inducing compressive forces in plane and subsequent instantaneous cell crowding. Moreover, the low-profile device is completely portable and can be mounted directly onto an inverted optical microscope. This enables visualization of the morphology and dynamics of living cells in stretched or compressed conditions using a wide range of high-resolution microscopy techniques.

METHOD SUMMARY

We used 3D printing technologies to create a mechanical testing device that enables long-term culture of cells on pretensioned substrates that can be returned to zero stress under user command. The devices are compatible with a variety of imaging platforms, enabling real-time visualization of the dynamic response of cells to in-plane compression.

KEYWORDS

3D printing • cell compression • epithelial mechanics • mechanobiology • microscopy

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Epithelial homeostasis requires the precise regulation of cell density and has been found to be controlled by mechanical intercellular forces within the monolayer [1]. In particular, when crowded above the target density, cells experience intercellular compressive forces and undergo mechanically influenced live-cell extrusion [1–5]. Failure to extrude can result in tumorigenesis, while hyper-extrusion can compromise barrier function. Therefore, studies of epithelial cell mechanics and mechanobiology are critical to understanding healthy tissue maintenance and also how dysfunction of cell migration can lead to cancer and other diseases [6].

Unfortunately, there are limited experimental tools to enable such studies. Several commercial cell-stretching systems are available (e.g., from Flexcell International, NC, USA; Strex, CA, USA; CellScale, Ontario, Canada) and a number of custom-built devices using extensible silicone [7–12], piezoelectric [13], pneumatic [14–17] and recently dielectric actuation [18] have been reported. However, microscopy studies of crowding-induced live-cell extrusion are particularly demanding: cells must be cultured on stretched membranes for hours to days to reach confluency before crowding is induced and then must be imaged at high spatiotemporal resolution to observe their morphology and dynamics. Although some current devices are amenable to imaging [8,10,11,15,16,19], it is not possible to maintain loading conditions continuously in culture or to move the cells in a loaded state between the incubator and the microscope. This severely limits analysis of the dynamic changes that occur before, during and after cell extrusion.

Here, we report a new approach based on the design of low-cost and completely portable cell stretching devices that allow culture of cells on pretensioned substrates and that can be easily transferred to imaging platforms without any changes in cell loading. Moreover, the ease and low cost of manufacturing (typically <$300) allows for the simultaneous use of multiple devices.

To meet the needs of dynamic extrusion measurements, we applied the following constraints: the device must be: compatible with the existing microscope stage and apertures (here, the Leica TCS SP8 laser scanning confocal fluorescence microscope); capable of imposing uniform, repeatable compressive strains up to 30%; able to be placed within a cell incubator and easily transported to/from the microscope facility; liquid-tight to ensure that culture medium does not leak during transport or imaging; and amenable to sterilization. Two additional constraints are that the device must position the cells within the desired focal depth and imaging must occur directly through a glass coverslip.

To customize the device, we employed in-house fused deposition modeling style 3D printing with a Stratasys f270 printer. F123 acrylonitrile butadiene styrene build material was chosen due to its high resistance to thermal deformation, ease of printing and cost–effectiveness. Quick support release was used as the support material. Following printing of the necessary device components, the device was assembled per the instructions provided in the Supplemental materials. All components were purchased from McMaster-Carr or 3D printed, except for the double-sided lead screw, for which a portion of the purchased threaded rod was manually reduced and rethreaded.

In operation, the user first secures both ends of a rectangular silicone substrate between the two clamps (Figure 1), while threading the central portion of the substrate over the indenter. The indenter serves as a height reference for the substrate position, ensuring that the cells, which are adhered to the bottom surface of the substrate, are positioned within the objective focal depth. Several fiducial markers spaced 20 mm apart were engraved into the base of the device to allow the user to quickly apply a predetermined strain to the substrate. To

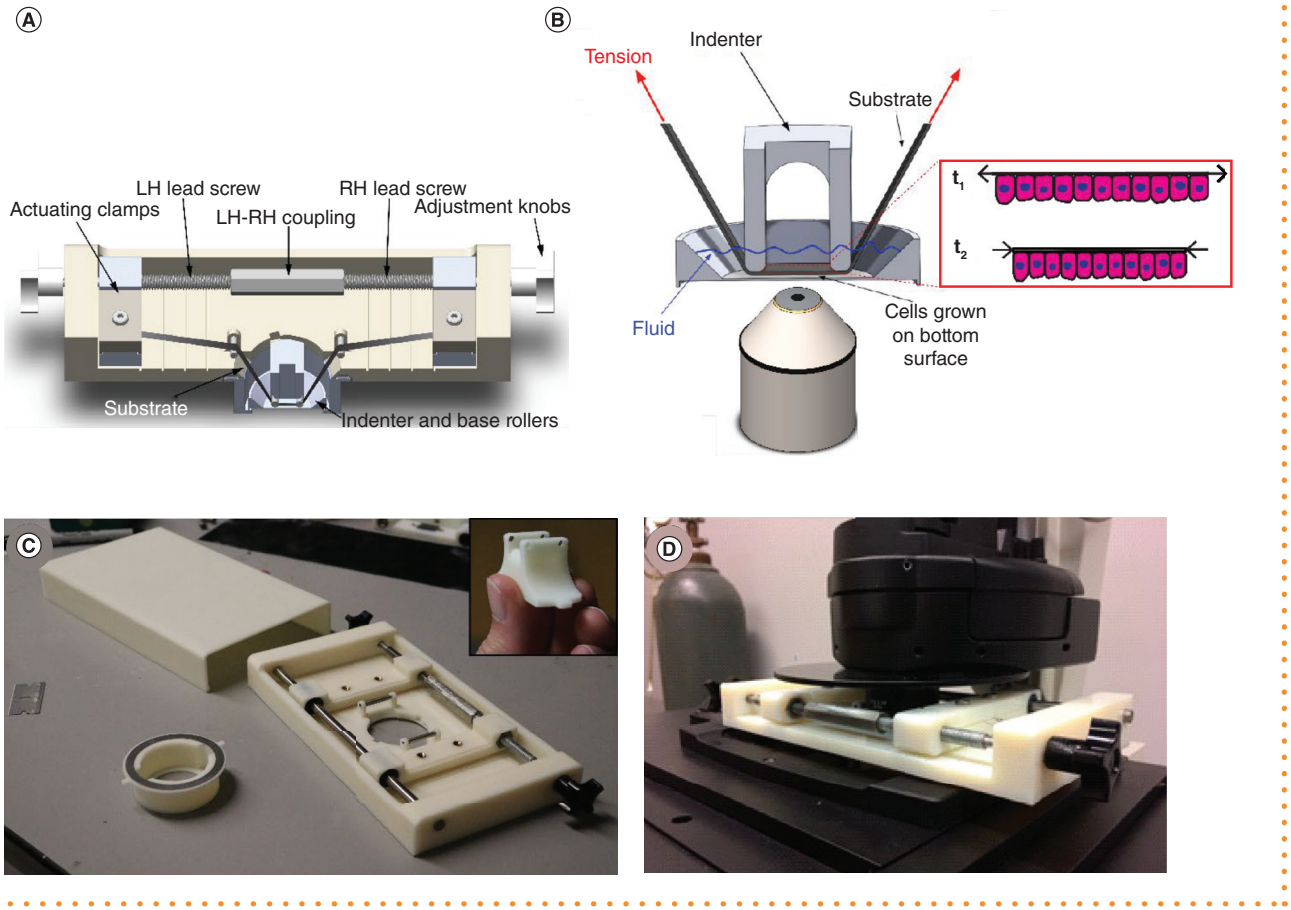

Figure 1. Device design and construction compatible for long-term cell culture and live-cell imaging. (A) Schematic of device in cross-section with the substrate in tension. (B) Lead screw rotation induces substrate compression. (C) Image of assembled device with custom fit lid. (D) Device mounted on a common stage holder of an inverted fluorescence microscope in operation.
LH: Left hand; RH: Right hand.

provide an aqueous environment for the epithelium, a 35-mm glass-bottomed dish (MatTek) containing medium was held magnetically by a 3D-printed holder just below the substrate.

Tension within the substrate can be varied by turning a double-sided lead screw, ensuring that both clamps move outward (or inward) simultaneously and that cells located in the imaging region are not significantly laterally displaced during testing. On the opposite side of the device, a guide rail serves as a support for the actuating clamps. Linear bearings between the clamps and the guide rail provide smooth motion while two sets of stainless-steel rollers supporting the substrate reduce friction to improve uniformity of the applied strain. The lead screw rotation is fully reversible, providing compression or tension to the substrate as desired. To ensure self-containment and minimize contamination, a lid can be attached to the top side of the device. Images and schematics of the completed device are provided in Figure 1; see Supplementary materials for additional schematics.

To determine the extent and uniformity of the strain within the membrane, we used digital image correlation to visualize the strain field (Figure 2). A random uniform distribution of speckles was spray-painted onto the silicone rubber; the painted strip was then loaded into the device and imaged under various states of strain. We measured proportional strain levels respective to revolutions of the lead screw, which averaged into a linear relationship across different strain states (Figure 2C). Full experimental details are provided in the Supplementary materials.

To demonstrate the utility of this device for measurements of cell dynamics under compression, we used a model epithelial system of Madin–Darby Canine Kidney II cells expressing eGFP E-cadherin [20]. To promote cell attachment, the silicone rubber substrate was first functionalized with fibronectin (see Supplementary materials for details) before being placed within the cell compression device and pretensioned as desired. Once the functionalized substrate was pretensioned, the device was inverted and approximately 50,000 cells were deposited onto the surface over the indenter. To ensure cell adherence, the inverted device was then covered with parafilm and placed in the incubator for 3 h. After incubation, the device was flipped and the adhered cells were rinsed with phosphate-buffered saline before being submerged in the medium-filled MatTek dish. The custom 3D-printed lid was then fitted to the device, which was transported to the incubator for culture until confluency was achieved (usually after 1–2 days).

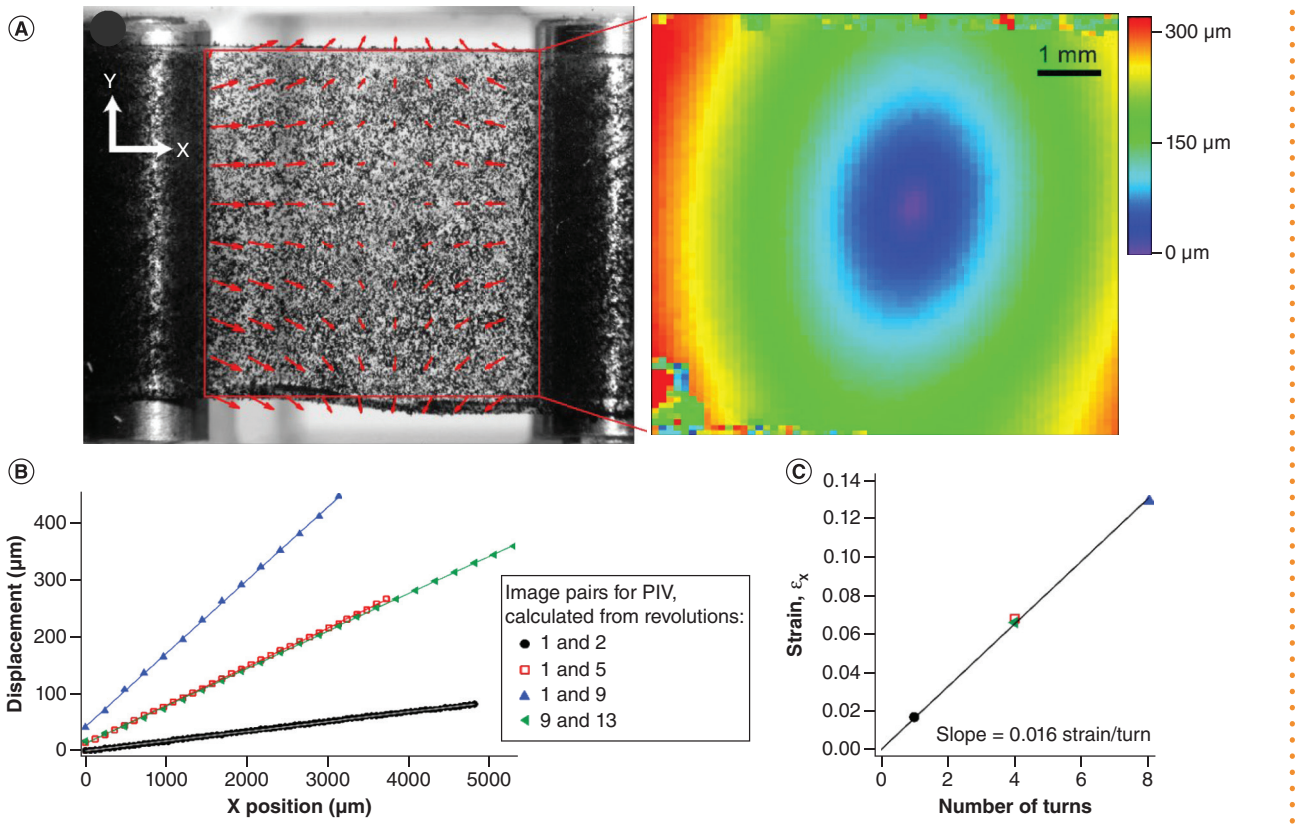

Figure 2. Digital image correlation demonstrates and quantifies linear strain of substrate. (A) Representative mapping of pixel displacement direction (left, arrows in foreground) and magnitude (right) obtained through the analysis of images of the speckled silicone rubber substrate (left, background). (B) The average lateral displacement as a function of x-position was quantified through the pairwise comparison of images obtained across different tension states. The numbers in the legend refer to the number of revolutions of the outer knob turning the lead screw (see Supplementary materials for details) for the two images forming the pair; the slope of each line gives the dimensionless substrate strain, e_x, per turn, shown in (C).

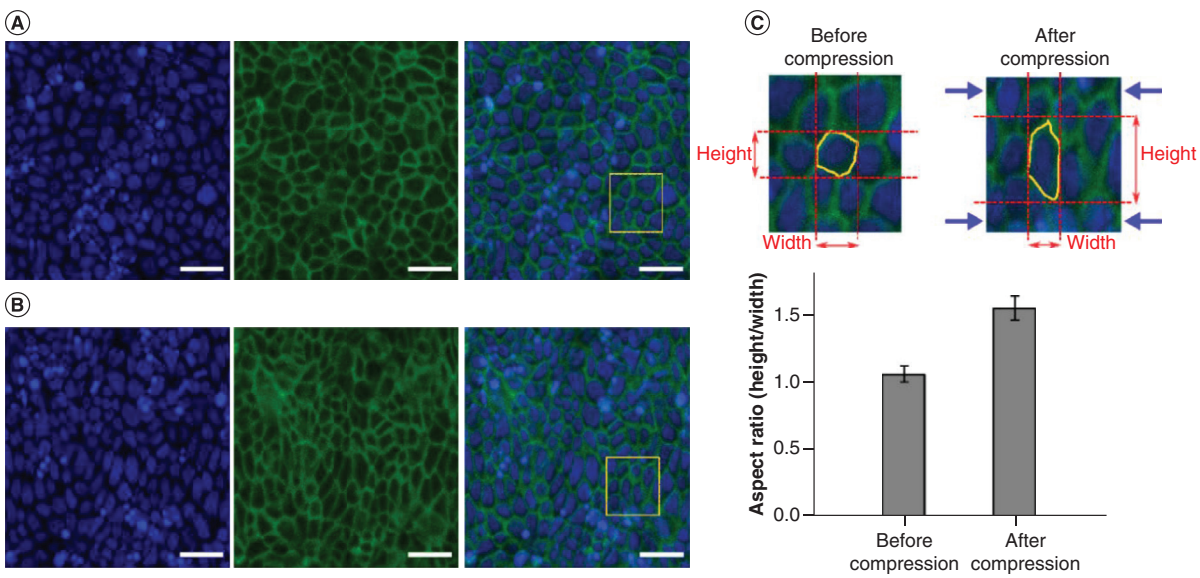

Figure 3. Substrate compression successfully crowds cells for live-cell extrusion studies. (A) Uncompressed and (B) compressed epithelial cell monolayer cultured on a pretensioned (prestrain value of 30%) silicone rubber substrate within the 3D-printed compression device (nuclei: blue, Hoechst; E-cadherin: green, eGFP; far right are merged channels). Scale bar is 30 µm. (C) Zoomed-in regions of cells before and after compression. Bar graph shows mean values of aspect ratio as measured from 40 randomly selected cells from each of panels A and B. Error bars indicate standard error of the mean.

Once cells reached confluency, the device was transferred to the microscopy facility, where the tension was released by the desired amount and the compressed cells were imaged using an inverted Leica TCS SP8 spectral confocal microscope with a 10× air objective (Figure 3). Before compression, the cell aspect ratio (height/width) was 1.07 ± 0.06, indicating no preferred orientation within the plane of the substrate. Upon compression (30% strain), the aspect ratio increased to 1.56 ± 0.09, due to a reduction in cell size along the compression axis and an elongation in the orthogonal axis. We also measured an approximate 20% increase in cell density along the compression axis (Supplementary Figure 1).

In summary, we designed, manufactured and tested a low-cost portable cell compression device amenable to both long-term culturing and imaging by microscopy. This innovation enables visualization of the morphology and dynamics of living cells in stretched or compressed conditions using a wide range of high-resolution microscopy techniques.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0160

AUTHOR CONTRIBUTIONS

AH Khankhel and MT Valentine conceived of the project. J Abram and LP Dow designed, manufactured and tested devices. LP Dow and AH Khankhel performed biological assays. MT Valentine supervised the project. LP Dow and MT Valentine analyzed data and LP Dow, MT Valentine and J Abram wrote the manuscript.

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FINANCIAL & COMPETING INTERESTS DISCLOSURE

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Reports

BioTechniques

Organoid model of urothelial cancer: establishment and applications for bladder cancer research

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ABSTRACT

3D cancer cell models are suitable for drug evaluation because they more precisely mimic tissue architecture than 2D cultures. To study cytotoxicity of anticancer agents, the most sensitive CellTiter-Glo 3D assay is used. However, this is an end point assay, so it is not possible to consider the variance of the starting material amount in the final reading. It is difficult to maintain an even plating density of 3D organoids for cytotoxicity analysis. We present a simple, 3D bladder cancer culture that can be maintained, cryopreserved and used for molecular and drug response studies. We applied a simple modification of the drug response assay for 3D cultures by measuring the background signal with the CellTiter Blue assay before drug application.

METHOD SUMMARY

We describe a simple, reliable bladder cancer 3D organoid culture. We modified the drug response assay for 3D cultures. The widely used assay CellTiter-Glo (Promega, WI, USA) is an end point application, so it is not possible to measure signal before and after treatment for the same samples; rather, it is necessary to use a separate set of samples as a control and it is challenging to plate cells for 3D organoids evenly in control (untreated) and experimental samples. The CellTiter Blue assay allows measurement of fluorescence before and after treatment for the same sample set. The measurement of untreated cells is counted in the final fluorescence analysis of the samples after drug treatment and diminishes the effects of uneven cell plating. We also utilized other modifications to the previously described maintenance and analysis of these 3D cultures.

KEYWORDS

3D cell culture ● bladder cancer ● cell toxicity assay ● drug response ● organoid

Bladder cancer (BC) is a common malignancy with a relatively poor outcome. Superficial low-grade urothelial carcinomas of the bladder represent 70% of all bladder tumors and recur often, whereas muscle-invasive BC progresses rapidly and poses a significant risk of mortality [1]. In the USA, 80,470 new cases of urothelial carcinoma were diagnosed in 2019 and 17,670 deaths were associated with this disease [2]. Recent advances in the molecular characterization of BC deciphered intrinsic subtypes that responded distinctly to tested therapeutics and were associated with different prognoses [1]. This variability indicates that a better understanding of the molecular features of individual tumors and the application of specific therapeutic modalities for definite situations (personalized medicine) are highly demanded.

Generation of reliable models of BC will help to achieve these aims. There are currently a limited number of BC model systems that provide a realistic presentation of the normal urothelium and BC [3].

Established 2D adherent cell cultures of BC have been the primary tool for cancer biology research and have been used intensively in preclinical studies. These systems are relatively easy to maintain and analyze; they are therefore convenient for laboratory investigation. BC cell lines reflect some of the genetic aberrations found in human BC and display numerous features typical of malignant cells [4]. However, there are some limitations to the exploitation of this system in cancer biology, notably the absence of the typical tumor 3D architecture and surrounding environment (extracellular matrix, supportive and immune tissue). This limits the value of results obtained through *in vitro* investigations with a 2D culture of BC cells.

3D culture of malignant cells mimics the tumor environment better than 2D cultures, even when grown as a suspension without the addition of extracellular components. The addition of the extracellular matrix to these cultures is expressed in a more realistic presentation of the original tumor [5,6].

The type of 3D cellular self-assembled structures formed from a single cell – possibly a stem cell – is thought to closely recapitulate the *in vivo* environment and is defined as an 'organoid'. As a functional unit, an organoid is composed of multiple cell types and contains multicellular organ structures, mimicking the tissue of origin and possessing multiple features of the original tumor [5,6]. Therefore

3D organoid cultures derived from the tumor tissue improve our understanding of the disease biology [6]. For BC, such systems were generated by several groups via the production of 3D structures from established cell lines [7,8] and the development of organoid cultures from patient-derived BC tissue [3,8,9]. The 3D organoid cultures derived from BC patient tissue closely represented the original tissue genetically and histologically and were used effectively for molecular studies and preclinical anticancer drug testing [3,6,9,10].

Here we introduce a long-term culture system for human BC cells consisting of 3D structures of epithelial cells derived from patients and established cell lines, using modified growth conditions and simplified media composition. These structures recapitulate many aspects of the original tumor; they can be maintained for several months (five to six passages), cryopreserved for future investigation and used for animal studies. These BC organoids are also convenient models for biochemical, molecular studies and the preclinical testing of potential therapeutic modalities. Drug response analysis can be performed with the application of the modified cell viability assay. This assay allowed us to adjust the fluorescence signal based on the original organoid plating and to test drug efficiency at different time points during drug treatment.

Materials & methods

Cell lines & reagents

Established cell lines

Human BC cell lines RT4, 5637 and T24 were purchased from the American Type Culture Collection (VA, USA) and maintained in RPMI 1640 (Thermo Fisher Scientific, MA, USA) containing 10% fetal bovine serum, glutamine and penicillin/streptomycin. Cells were cultured at 37°C under 5% CO_2. Cell lines were tested for mycoplasma and authenticated by short tandem repeat analysis by the American Type Culture Collection. RT4 cells maintain the transitional epithelial appearance, are highly differentiated and derived from recurrent transitional cell carcinoma of low grade; the 5637 cell line was generated from primary grade II BC and formed tumors in nude mice; the T24 cell line was generated from highly aggressive undifferentiated and invasive transitional cell carcinoma and also formed tumors in nude mice. Reagents whose source is not specified in the following sections were purchased from Sigma-Aldrich (MO, USA).

Primary culture of urothelium cells

Primary cultures were established from surgical samples of normal urothelium obtained from ureters during kidney transplantation surgery. The Institutional Review Board approved the collection and analysis of all samples and written informed consent was obtained from each subject. Cell culture was initiated and cultures maintained as described previously [11,12]. Briefly, the urothelial layer was mechanically detached from the ureter and digested with TrypsinLE. Cells in the form of a single-cell suspension were plated in six-well culture plates and maintained as described. Primary urothelial cells (PUC) were used up to the fifth passage.

BC spheroid & organoid preparation

Multicellular spheroid preparation from cell lines

Spheroids from cell lines were prepared by an aggregation-based method [13]. Briefly, 1×10^6 or 1000 cells (PUC, RT4, 5637 or T24) were seeded into related full growth media in 60-mm or 96-well plates, respectively; the plates were coated with poly-2-hydroxyethyl methacrylate (Sigma-Aldrich, MO, USA). Spheroids were formed 24 h after seeding and used in further experiments. For drug sensitivity experiments, single cells were embedded in 20 μl of Cultrex Basement Membrane Extract that was placed on the bottom of untreated 96-well plates. Polymerized Basement Membrane Extract with embedded cells was covered with 0.1 ml of growth media (RPMI 1640 + 10% fetal bovine serum supplemented with antibiotics and glutamine).

Primary 3D cultures generated from surgical BC samples: cancer tissue-originated spheroids/organoids

Human bladder tumor specimens were collected from patients diagnosed with BC and treated with transurethral resection at the Department of Urology at Stony Brook. Because our current organoid lines were established from transurothelial resection of bladder tumor specimens, they were enriched for nonmuscle-invasive bladder tumors, which represent the majority of urothelial cancers. In this study, we used five organoid cultures developed from low-grade nonmuscle-invasive tumors of stage Ta or T1. S1, S2, S3 and S4 cell lines were tested at passage 1–2. The organoid line S2-1 was started from a previously frozen S2 sample (at passage 2). This study was approved by SUNY at Stony Brook Institutional Review Board committee and written informed consent was obtained from all patients involved. All tissue samples were examined by a dedicated uropathologist.

Cancer tissue-originated spheroid (CTOS) preparation and culture were performed as described previously, with some modifications [13]. The approach was based on the partial preservation of intercellular junctions between urothelial cells during mild mechanical and enzymatic digestion with Liberase™ DH (dispase high).

Briefly, primary tumors were minced finely in the tube using a pair of small scissors and digested enzymatically for 2 h using Liberase DH (Roche Applied Science, WI, USA) in advanced Dulbecco's modified Eagle medium/Ham's F-12 (Thermo Fisher). ROCK inhibitor (Y-27632, 10 μM) was always added to the starting culture and markedly increased culture survival, as indicated [14]. After passing the resulting mixture through filters and cell strainers, the cell fractions retained on the strainers with mesh 40 μm – termed the organoid fraction – were collected.

The organoids were cultured with CTOS medium (advanced Dulbecco's modified Eagle medium/Ham's F-12, B27 [2% Thermo Fisher 17504001], A83-01 [5 μM], N-acetylcysteine [1.25 mM] and nicotinamide [10 mM]) [3]. For CTOS maintenance we add the combination of FGF10 (100 ng/ml of PeproTech 100-26), FGF7 (25 ng/ml of PeproTech 100-19), FGF2 (12.5 ng/ml of PeproTech 100-18B) and HER3 100 ng/ml from PeproTech (NJ, USA).

For growth and cytotoxicity assays, CTOSs were further digested with TrypsinLE and additionally mechanically disrupted by pipetting. Single cells were resuspended in 20 μl of BME and placed into wells of a 96-well plate. When the BME was solidified, CTOS medium was added. Organoids were frozen in freezing medium (50% fetal bovine serum, 10% DMSO) and 40% organoid medium and could be recovered efficiently as described [3].

Immunofluorescence microscopy & whole mount preparation

Organoids were released from BME with cold Organoid Harvesting Solution (3700-100-01 Cultrex BME) and fixed in a 4% formaldehyde for 3 h. Fixed organoids were blocked with 2% donkey serum in phosphate-buffered saline. Blocked organoids were permeabilized with 0.5% Triton in phosphate-buffered saline and incubated with primary antibodies at 4°C overnight. For this study, we used E-cadherin antibodies (#3195 Cell Signaling Technology, MA, USA). After incubation with the primary antibody, organoids were washed and incubated with related fluorescently labeled secondary antibodies and DNA was stained with DAPI. Phalloidin (Molecular Probes Alexa Fluor™ 594–phalloidin conjugate) was used for F-actin labeling. Organoids were mounted with ProLong Gold Antifade Reagent (Cell Signaling Technology). Images of fluorescent cells were obtained with a Zeiss LSM 510 META NLO two-photon laser scanning confocal microscope system.

Cell viability assays

The assay utilized in the current study is the CellTiter Blue assay (Promega, WI, USA), which is based on the fluorescence reading of resorufin converted by cell enzymes from resazurin and allows measurement of the signal from untreated organoids. After washing out the CellTiter Blue reagent, we can add drugs and then add the reagent again to detect a final signal after treatment. The variation of the primary cell number can be counted using the first measurement. For the drug response assay, organoids were plated into ultra-low-attachment 96-well plates (Corning, NY, USA) in BME with similar density as described in the previous section. After 2 days, organoids were ready for drug toxicity testing and cell viability was tested using the CellTiter Blue assay. Wells with organoids were randomly selected (in triplicates). Drugs were added at the indicated concentration (4–7 points) in dedicated wells and cells were incubated for 3 days. Reference reading (without treatment) was performed in two ways. First, a separate set of wells with organoids, randomly selected, was used to produce a detection reference signal. Second, a reference reading was obtained from wells assigned for treatment. The CellTiter Blue reagent was washed out and drugs were added. The final reading was performed in the same wells after treatment. Two reference signals were used for two different calculations of cell viability.

Drug selection & dose rationale

Drugs were selected based on: current application in the clinic for BC treatment (cisplatin [CDDP], gemcitabine, mitomycin C); potential use for BC treatment (dasatinib) [15,16]; or the previously published data [9] and pilot experiments with BC cell lines (Chk1/2, PI3K, mTOR and HSP90 inhibitors). For this study, CDDP, mitomycin C, gemcitabine and dasatinib were obtained from NIH Cancer Drug Depository; tanespimycin (HSP90 inhibitor), NVP-BEZ235 (dual PI3 kinase and mTOR inhibitor) and PF477736 (Chk 1/2 inhibitor) were purchased from Cayman Chemical.

Data presentation & statistical analysis

Data analyses were performed using GraphPad Prism 6.01 Software (GraphPad, CA, USA) and the values of IC_{50}, Hill slope and AUC were calculated by applying nonlinear regression (curve fit) and the equation log (inhibitor) versus normalized response (variable slope) [9].

Results & discussion

Spheroid & organoid morphology & growth dynamics

Spheroids were formed using aggregation conditions or were grown in BME from single cells using medium that was used for 2D culture.

At 3D culture conditions, the organoid and spheroid lines (Figure 1A) displayed morphologies ranging from spheroidal (a, b, d, e) and grape type (c) to relatively loose aggregates of cells (f). This is typical for cells derived from more aggressive tumors (f T24 3D cellular spheroids). The average success of organoid establishment was approximately 70%.

We tested several growth conditions for BC organoids. For primary growth of multicellular spheroids from established cell lines, we used plating for aggregation in untreated culture plates; for CTOS growth, they were embedded in BME.

As described earlier, the organoid growth medium contained additives: FGF2, FGF7, FGF10, HER3 and ROCK1 inhibitor Y-27632; we did not use Noggin or R-spondin. In preliminary experiments we did not observe substantial growth preference with these additives (data not shown). Therefore we simplified the culture process based on the description for previously established BC derived organoid cultures [3,9,10] by using FGFs and HER3 as the only growth factors. These 3D cultures can be maintained for months, propagated and cryopreserved for future evaluation.

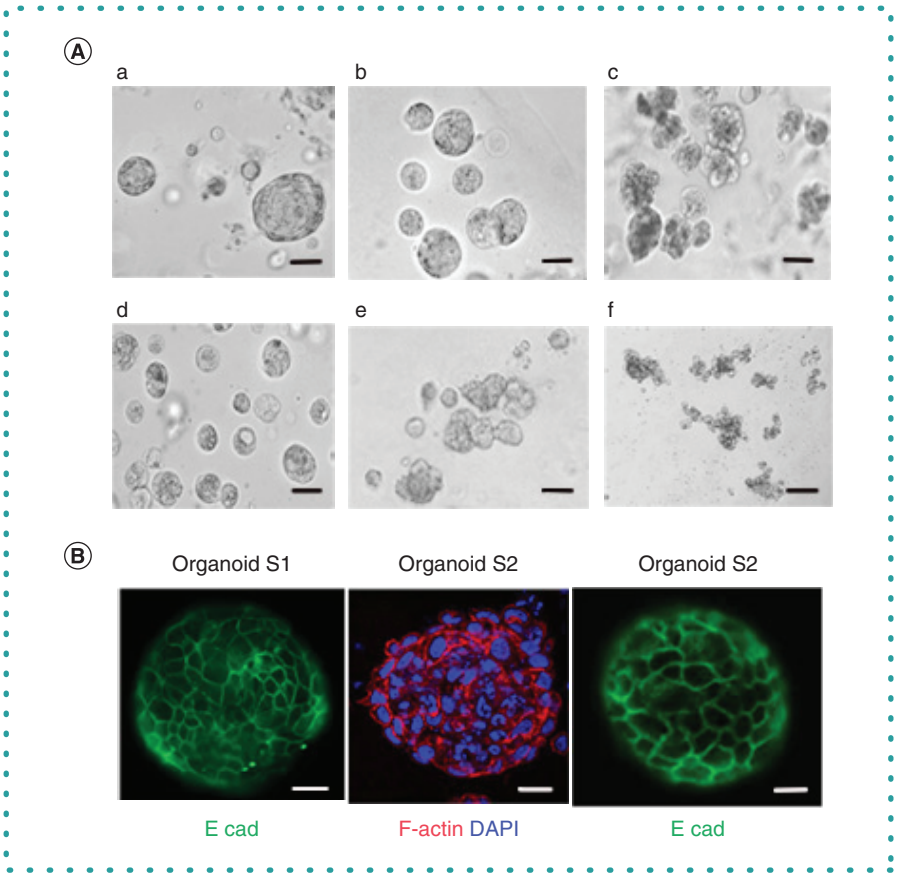

Figure 1. Patient-derived organoids and spheroids generated from BC cell lines. A) Patient-derived BC organoids (phase contrast) a. Sample 1. b. Sample 2. c. Sample 3. d. RT-4 spheroids. e. 5637 spheroids. f. T24 spheroids. **(B)** Immunostaining of organoids S1 and S2 for indicated markers (whole mount preparation, confocal microscopy).
Scale bars: 150 μm for part A and 75 μm for part B.
BC: Bladder cancer.

Immunocytochemical evaluation of organoids

We did not compare the sequencing profile of organoids with that of the original tumor in this study; however, the technique developed for CTOS generation allowed us to grow cultures that preserved some features of the original tumor as described [17]. Organoids described in this study were derived from low-grade nonmuscle-invasive tumors that conserved many features of normal urothelium. Patient-derived organoids (S1 and S2) showed the presence of E-cadherin, located on intercellular borders (Figure 1B, left and right panels). The formation of organized spheroidal structures with regular distribution of E-cadherin on the cell borders indicated that cells forming organoids also showed properties of urothelium. The molecular similarity of organoids with the tissue of origin has been shown in many organoid studies, including some of BC-originated organoids [9,18].

Confocal microscopy regularly demonstrated that junctional F-actin was significantly organized at points of cell–cell contact throughout the spheroids, indicating that it stabilizes and strengthens intercellular adhesive interactions [3,19,20]. In addition, for cells in which nuclei occupy a large volume, F-actin distribution appears as border staining on optical sections (Figure 1B, center).

We used whole mount preparation in this study to simplify the staining procedure and to increase antigen survival during the preparation procedure (as compared with immunohistochemistry with paraffin sections). For the whole mount preparation, we used longer incubation with primary antibodies at higher concentration (always overnight and five-times more concentrated than for labeling of paraffin sections). Adjustment of the staining conditions allowed us to get even, deep immunolabeling of organoids.

Cell viability assay

The major application of cancer cellular models is their ability to reproduce clinical conditions in response to drug treatment. The assay that is most commonly used in organoid studies is CellTiter-Glo 3D (Promega) [3,9]. This assay provides the highest sensitivity with a low background when compared with other cell viability assays; it can detect 50 cells in a well of a 96-well plate, whereas CellTiter Blue (fluorescence reading-based assay) needs 360 cells for a measurable signal. The number of cells per well in organoids is usually higher than 300–500; therefore the CellTiter Blue assay is sensitive enough for this aim.

Table 1. Cell viability (fluorescence signal) from organoids in untreated samples and in a treated set before and after treatment.

	Exp 1		Exp 2		Exp 3	
	M	SD	M	SD	M	SD
Untreated cells (RFU)	58,554	4932	42,917	4209	65,218	2218
Experimental cells before treatment (RFU)	45,403	2211	57,581	3119	43,746	874
Experimental cells after treatment (RFU)	58,554	4932	42,917	4209	65,218	2218

Results of three experiments for CDDP action (75 μM) on urothelial organoids (S2). The CellTiter Blue assay was used. Three random wells of 96-well plates were used as untreated controls and fluorescence reading was measured at the end of the experiment. Fluorescence of organoids from other three wells were measured before and after CDDP treatment (mean ± SD).
CDDP: Cisplatin; M: Mean; RFU: Relative fluorescence units; SD: Standard deviation.

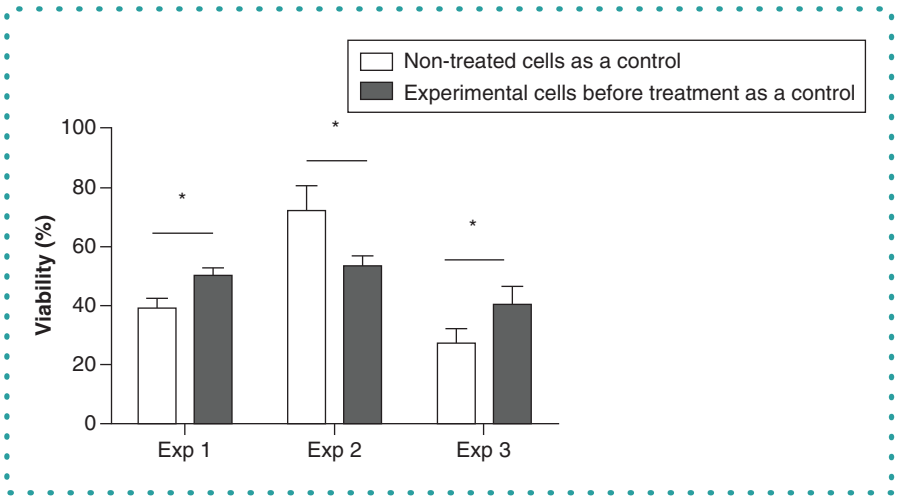

Figure 2. Determining the exact amount of starting material for results of drug response assay with 3D urothelial organoids. Viability (%) was calculated in two ways, using either separate untreated or CDDP-treated cell sets before addition of the drug as a reference. The Student *t* test was performed to compare viabilities at two methods for all three experiments.
***p < 0.05.**
CDDP: Cisplatin.

Unlike 2D adherent culture, plating BME-embedded 3D cellular structures evenly in wells is not always conceivable. Although, before plating, cells can be trypsinized to a single-cell suspension and counted to allow plating an equal number of cells per well, some dispersion of the starting amount of biological material always exists and measuring the amount of starting material in different wells is necessary. To evaluate the amount of starting material before using an end point assay like CellTiter-Glo 3D, it is necessary to use some approach to estimate cell numbers in different samples (wells). Several researchers overcome this by counting and measuring organoids in wells before treatment [1,10,21]. However, microscopic methods are always subjective and demand analysis of large sample numbers and the use of a scoring system and image software, like ImageJ (National Institutes of Health, MD, USA) [1]. In addition, not all cells forming organoids may be viable at the starting point. Another option is the use of a vital dye assay (CellTiter Blue) for normalization. This approach was applied before the following MTT (end point) measurement [21]. The procedure described herein utilized the only reagent that can be used for testing cell vitality before treatment and easily washed out after fluorescence detection without any toxicity to cells (incubation time 1 h). This procedure can be repeated at several time points to produce a time curve, if necessary.

In this study, we used vital staining with resazurin (CellTiter Blue) that allowed us to measure the original number of viable cells in the formed organoids/spheroids before treatment. After a complete wash of the staining reagent, drug treatment can be started and at the end of the treatment, another CellTiter Blu test can be performed. The variance in cell viability was detected between organoids in one set and between control and experimental sets of the samples (Table 1). When two versions of the CellTiter Blue test were performed, a difference in the final viability results was observed (Figure 2). Three independent experiments displayed different drug toxicities when separately assigned treatment sets of cells were used as a reference.

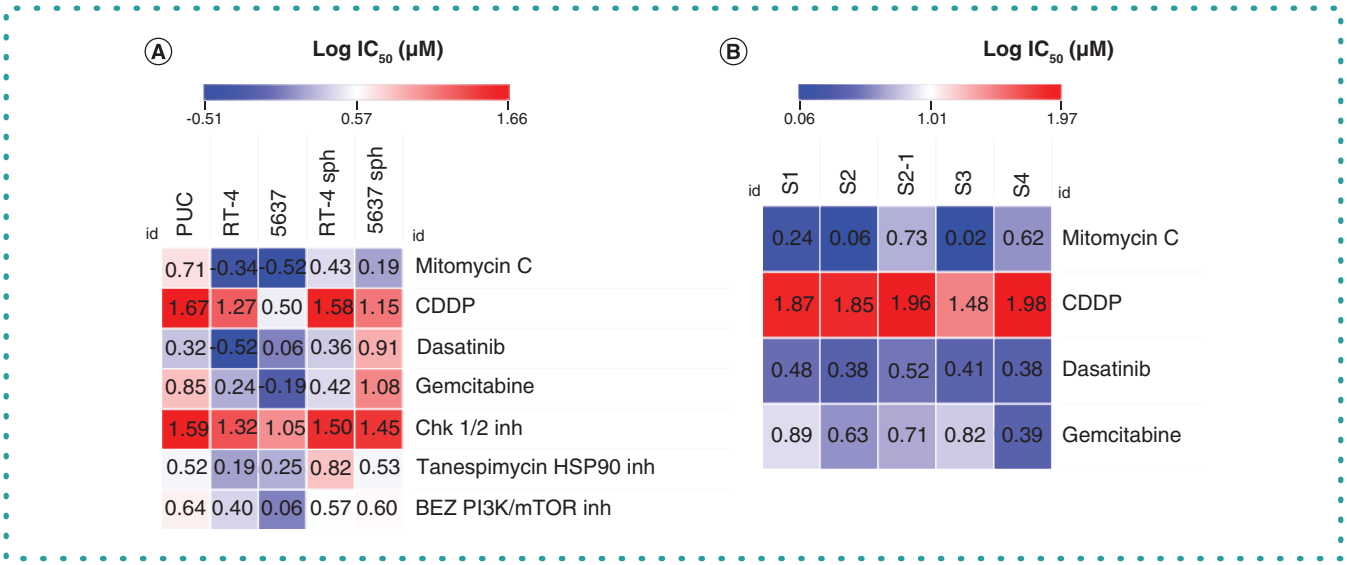

Figure 3. Heatmap of log IC_{50} values calculated from drug response analyses of 2D cell lines, related 3D spheroids and patient-derived organoids by applying nonlinear regression (curve fit).

Drug response of organoid lines

To examine organoid lines as preclinical models for evaluation of drug response, we performed dose titration assays to examine the effects of the tested compounds. To this aim, we used five organoid lines (S1, S2, S2-1, S3, S4), PUCs, two established BC lines in 2D form (RT-4, 5637) and 3D spheroid cultures generated from these two lines.

PUC was more resistant to all tested drugs than BC-originated cell lines in monolayers. RT-4 responded better to dasatinib then 5637 cells. CDDP, mitomycin C and gemcitabine were more toxic to 5637 than to RT-4 cells. Generally, the spheroid culture of RT-4 and 5637 cells responded less efficiently than related 2D cultures (Figure 3A).

We also observed variability in the response of individual organoid cell lines to the distinct drugs. It is notable that S2-1 organoids derived from the second generation of organoid S2 were more resistant than S2 to all four chemotherapeutic agents tested (Figure 3B).

The assay applied for drug response testing with organoids and spheroids showed typical results for the drugs and cell types used [9,16]. Spheroids derived from 2D cell lines are more resistant than monolayer cultures (Figure 3A). This may be explained by more difficult conditions for drug delivery inside structures and by the better viability of 3D organoids because of 3D architecture and the presence of extracellular components.

Conclusion

We present a reliable simple system for generation, maintenance and analysis of 3D organoid culture for BC research.

Future perspective

Over the next decade, new cellular models – 3D tumor organoids – will enter the clinic for future molecular and genetic characterization of neoplasms and for testing new therapeutic modalities. Standardization and simplification of the establishment and characterization of such models is an important step for the successful application of personalized treatment of individual cancer patients.

Author contributions

T Whyard participated in the study design, performed the experiments, gathered data and cultured cells. W Waltzer and F Darras provided clinical samples and critical assistance to analyze the data. J Liu provided uropathology consultancy. V Romanov conceived the idea, revised and edited the manuscript and supervised the study.

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Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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A fast and simple fluorometric method to detect cell death in 3D intestinal organoids

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ABSTRACT

Organoids recapitulate the (patho)physiological processes in certain tissues and organs closer than classical cell lines. Therefore, organoid technology offers great potentials in drug development and testing, and personalized medicine. In particular, organoids can be used to study and predict drug-induced toxicity in certain tissues. However, until today few methods had been reported to analyze cell death in 3D-microtissues in a quantitative manner. Here, we describe a novel fluorometric method for the quantitative measurement of specific organoid cell death. Organoids are stained simultaneously with the cell impermeable nuclear dye propidium iodide and cell permeable Hoechst33342. While Hoechst allows in-well normalization to cell numbers, propidium iodide detects relative proportion of dead cells independent of hydrogel. Measurement and analysis time, as well as usability are drastically improved in comparison to other established methods. Parallel multiplexing of our method with established assays measuring mitochondrial activity further enhances its applicability in personalized medicine and drug discovery.

METHOD SUMMARY

We developed a fluorometric method to quantify cell death in intestinal organoids based on DNA staining for normalization and cell permeability for cell death. The method, independent of cell number over a wide range, can be used to study toxic effects of drugs on intestinal organoids or other 3D microtissues, and can be combined with photometric assessment of mitochondrial respiration.

KEYWORDS:

cell death • DNA dyes • fluorescence • intestine • organoids • personalized medicine

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Organoids grown from tissue-specific stem cells have become useful tools to study physiological and pathophysiological processes in an *in vitro* system much closer to the *in vivo* situation than cell lines. In 2009, Sato *et al.* described a method on how isolated primary intestinal crypt cells can be cultured over prolonged periods of time [1]. Today, organoids are being generated from nearly every tissue and any organism [2–4]. They are defined as organ-like structures, which self-organize in 3D. Comprised of several tissue- and organ-specific cell types, including stem cells, organoids are capable of fulfilling a variety of organ-specific functions, for example, excretion or secretion [5].

Intestinal organoids are being used to study not only basic intestinal physiology [6], but also pathophysiological processes, for example, TNFα-induced epithelial cell death during inflammatory bowel disease [7,8]. Moreover, intestinal organoids have been used to study host–pathogen interactions, for example, during Zika virus infection [9]. Importantly, intestinal organoids may also represent an unlimited source of transplantable tissue suitable for regenerative medicine. In a proof-of-principle study murine intestinal organoids were successfully transplanted into the severely damaged colon of mice suffering from inflammatory bowel disease. Organoids not only engrafted functionally into the colon but regenerated the damaged epithelium [10]. Tumor tissue-derived organoids also provide interesting tools to study tumor-specific drug responses as well as tumor diversity *ex vivo*, for example, in colorectal tumors [11,12]. Therefore, primary and tumor organoids are being and will be frequently employed for drug discovery [13] and toxicity testing, but also drug screening in patient-derived malignant tissue [14–16]. In this regard, patient-derived tumor organoid (PDOs) from pancreatic, prostate and gastrointestinal cancer are being used to model drug responses of patient-specific tumors in comparison to normal parental tissue [17–22]. This is of particular interest, as in gastrointestinal cancer it has been demonstrated that the parental and the PDO mutational spectra overlap up to 96%, on top of histological similarities [21].

Thus, by closely resembling the primary tumor *in vivo* drug testing in PDOs represents a further step towards personalized medicine [21]. Moreover, organoid-like 3D primary cell culture models are being effectively used to screen a large number of emerging oncology compounds for their cytostatic and cell death-promoting activity [23].

A significant problem of cell death screening in organoids is their 3D culture in extracellular matrix, which allows visual qualitative assessment of cell death, but impedes quantitative analysis of cell death. We have previously described the use of a modified MTT staining method to detect organoid survival, specifically, cell death in culture [24]. However, this method is strongly affected by mitochondrial respiration and only an indirect measure of cell survival, specifically, cell death. Other studies have used staining of dying organoids with propidium iodide (PI) and Hoechst33342 (Hoechst) to quantify cell death, employing complex and thus time-consuming high-content imaging [25].

The aim of this study was therefore to develop a simple, practical and quantitative method to study cell death in organoids using PI and Hoechst. While Hoechst is being used to normalize cellularity, PI uptake serves as a measure of cell death. The normalization allows sensitive cell death detection

over a wide range of cell densities. Fluorescence in 3D cultures can be quantified using a conventional plate reader. The increase of the PI signal relative to the constant Hoechst signal allows calculation of chemotherapeutic drug treatment-specific organoid cell death. Moreover, this method can be multiplexed with our previously described organoid-optimized MTT assay, allowing simultaneous analysis of respiration/survival and cell death. Thus, in comparison to other known assays our method offers a fast and simple protocol to detect organoid cell death in cell culture plates with minimal computational power and reduced overall costs.

MATERIALS & METHODS

Mice

C57BL/6 wild-type mice were bred and kept in individually ventilated cages at the central animal facility of the University of Konstanz.

Generation of intestinal organoids

Intestinal crypts were isolated as described previously with minor changes [1,24]. In brief, the small intestine of 8–16-week old C57BL/6 wild type mice was cut open longitudinally. Villi were removed by scraping with a microscope slide. Then, the intestine was cut into 3–4 cm pieces, washed three times with cold Ca^{2+}- and Mg^{2+}-free PBS, and incubated with 2 mM EDTA in PBS for 30 min at 4°C on a rotating wheel. All subsequent steps until seeding were performed on ice. Supernatant was removed and the tissue was filled up with fresh PBS. After shaking to remove residual villi, fresh PBS was replaced. This step was repeated and each fraction was checked for crypt/villus ratio under the microscope. Up to four crypt-containing fractions were pooled, filtered through a 70-μm cell strainer, centrifuged at 100 × g (3 min, 4°C) and resuspended in 5 ml PBS for crypt counting under the microscope. Numbers of crypts required for further culture were centrifuged at 80 × g (3 min, 4°C) and the pellet was resuspended in Matrigel (BD Biosciences) or in Basement Membrane Extract (BME) (Type II, R&D). A total of 200–300 crypts were seeded per well in 8 μl Matrigel or BME into a 96-well flat-bottom transparent cell culture plate (Sarstedt). Seeded crypts were incubated for 20 min at 37°C to let Matrigel and BME solidify. Then, 80 μl of complete crypt culture medium per well was added dropwise (Advanced DMEM/F12, 0.1% BSA, 2 mM L-glutamine, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM N-acetyl cysteine (Sigma), 1 × B27 supplement, 1 × N2 supplement (Gibco), 50 ng/ml mEGF, and 100 ng/ml mNoggin (Peprotech). hR-spondin-1 was added as conditioned medium of hR-spondin-1-transfected HEK 293T cells to a final volume of 25% (v/v) crypt culture medium. Organoids were cultured at 37°C in a 5% CO_2 atmosphere for 3 days before cell death induction.

Generation of tumoroids

Organoids from tumors (tumoroids) were generated as described previously with slight modifications [26]. Briefly, the small intestine of $APC^{Min/+}$ mice was opened longitudinally. Tumors were isolated from intestinal tissue with scissors and forceps and cut into small pieces. Subsequently, tumor fragments were washed three times with ice cold Ca^{2+}- and Mg^{2+}-free PBS, and incubated in digestion buffer (DMEM, 2.5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 200 U/ml Collagenase IV, 125 μg/ml Dispase II) for 1 h at 37°C, 5% CO_2. Tissue suspensions were shaken every 15 min. After 1 h tumor fragments were allowed to settle for 1 min. Subsequently, the supernatant was harvested and centrifuged at 200 × g for 3 min at room temperature. The pellet was resuspended in 5 ml PBS and filtered through a 70 μm and a 40 μm cell strainer. After centrifugation (200 × g for 3 min) cells were resuspended in 500 μl PBS and counted. Cell numbers were adjusted to 1.5 × 10^4 cells/50 μl Matrigel/BME. Complete growth medium with only 50 ng/ml mEGF was added. The culture medium was changed every 4 days. Tumoroids were split according to their density, but in general every week. Thus, medium was removed and tumoroids were incubated in cold PBS for 1 h on ice. Subsequently, Matrigel/BME was dissociated mechanically with a pipet tip and tumoroids were resuspended in cold PBS. Then, tumoroids were centrifuged at 200 × g for 3 mins and the pellet was resuspended in TrypLE Express (Thermo Fisher) for 15 min at RT. Tumoroid fragments were then centrifuged at 350 × g for 3 min and split in a 1:4 ratio for further culture.

Culture of human intestinal organoids

Human intestinal organoids were generated and cultured as described previously [27]. Frozen organoids were thawed and cultured in a mixture of 50% basal medium containing 500 ng/ml hR-spondin-1, 50 ng/ml mEGF, 100 ng/ml mNoggin, 10 nM [Leu15]-Gastrin I, 10 mM Nicotinamide, 500 nM A83-01 (TGFβ inhibitor), 10 μM SB202190 (p38/MAPK inhibitor), 10 μM Y-27632 (ROCK inhibitor) and 50% Wnt3A-conditioned medium. Growth medium was replenished every second to third day, and organoids were passaged weekly.

Staining of organoids with PI & Hoechst

Intestinal organoids in Matrigel/BME were stained with PI and Hoechst at a final concentration of 10 μg/ml each. Staining solution (dyes in PBS) was directly added to culturing medium after treatment. Organoids were stained for 30 min at 37°C, 5% CO_2 for subsequent analysis on the plate reader or by fluorescence microscopy. Then, staining medium was removed and replenished with fresh phenol-red free medium before analysis.

Fluorometric quantification of specific cell death in intestinal organoids

Cell death was induced in organoids and cell lines as indicated. Before measurement, staining medium was replaced with fresh phenol-red free medium. Stained organoids still embedded in hydrogel (Matrigel/BME) were measured in a plate reader (Tecan M200 Pro). Measurements were taken from the top. First, the gain was set to the wells for the highest expected cell death (PI) and the lowest expected cell death (Hoechst). Then, Z-position was determined automatically from the corresponding wells and was checked for values between 1.5 × 10^6 and 1.6 × 10^6 μm. Subsequently, fluorescence was measured with 25 flashes, with an integration time of 20 μs. Lag and settle time were set to 0 s. For each well, 4 × 4 measurements were taken with a border of 1 mm added around the measurement points. Excitation and emission wavelengths for PI were 535 m and 617 nm, respectively, and for Hoechst 361 and 486 nm, respectively. During the measurement, all wells were first measured for PI fluorescence and after a 30-s delay for Hoechst fluorescence.

Calculation of PI/Hoechst ratio & treatment-specific organoid cell death

The PI/Hoechst ratio was calculated by dividing PI by Hoechst RFUs:

$$\frac{PI}{H}ratio = \frac{RFU\ (PI)}{RFU\ (Hoechst)}$$

Using PI/Hoechst ratio, treatment specific organoid cell death was calculated:

$$treatment\text{-}specific\ organoid\ cell\ death\ [\%] = \left(\frac{x(sample)}{z(STS)}\right) \times 100 - y(ut)$$

Each sample was divided by the mean of all staurosporine (STS)-treated organoids and resulting values multiplied by 100. Then, mean of all untreated (ut) organoids was subtracted to set ut organoids to 0.

Determination of organoid viability & specific organoid death using MTT reduction

Organoid viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction as described in [24]. Briefly, after cell death induction MTT solution was added to the organoid culture to a final concentration of 500 µg/ml and incubated for 1 h at 37°C, 5% CO_2. Then, medium was discarded and 20 µl of 2% SDS solution in H_2O was added to solubilize the hydrogel (Matrigel/BME) for 1 h at 37°C. Subsequently, 80 µl of DMSO was added and incubated for 1 h at 37°C to solubilize the reduced MTT. The optical density was then measured at 562 nm in a plate reader (Tecan M200 Pro).

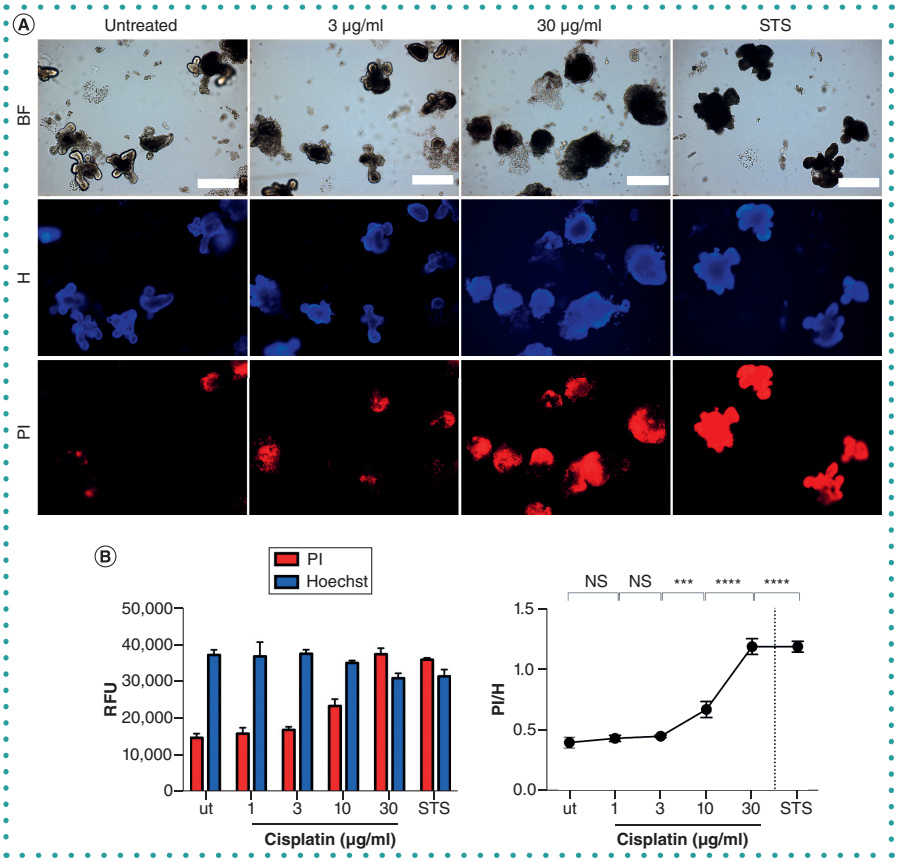

Figure 1. Quantification of PI and Hoechst in 3D organoids. Organoids were treated with indicated concentrations of cisplatin overnight. Then, organoids were stained with PI and Hoechst, and fluorescence was detected. (A) Representative BF and fluorescence microscopic images of untreated, 3 µg/ml cisplatin, 30 µg/ml cisplatin, and STS (5 µM) treated organoids (scale bar = 200 µm). (B) Left side: RFUs of PI and Hoechst over all concentrations. Right side: ratio of PI/H over the whole dose response. Mean ± standard deviation; n = 3 with technical triplicates. ***p ≤ 0.001; ****p < 0.0001.
BF: Brightfield; H: Hoechst; NS: Nonsignificant; PI: Propidium iodide; RFU: Relative fluorescence unit; STS: Staurosporine.

Quantification of intracellular ATP

Intracellular ATP was quantified with the CellTiter-GLo® 3D cell viability assay according to manufacturer's protocol (Promega). Briefly, after treatment medium was removed and cells were lysed in 100 µl pre-warmed CellTiter-GLo 3D reagent. Then, samples were incubated for 30 min on an

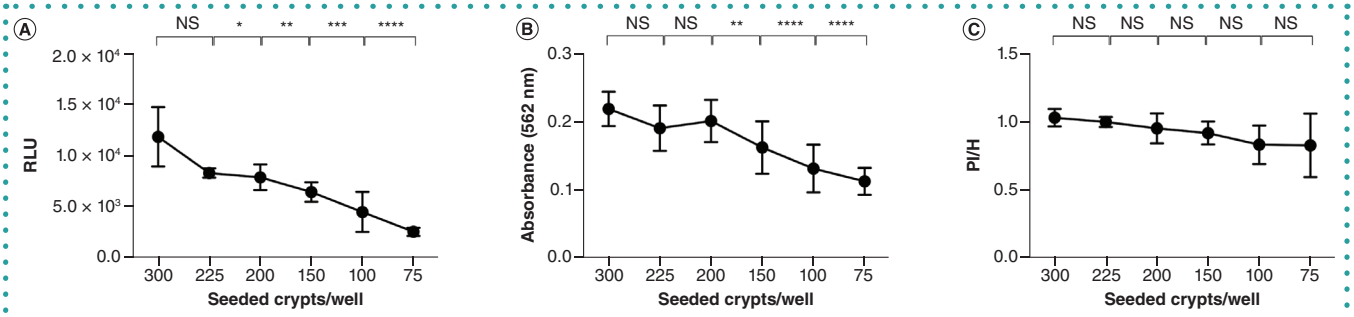

Figure 2. Assay comparison with different organoids densities. Murine intestinal crypts were seeded in indicated concentrations and grown for 3 days into organoids. Then, organoids were analyzed using CellTiterGlo (A) or MTT reduction assay (B). For PI/H fluorescence quantification (C), organoids were treated with 10 µg/ml cisplatin overnight before measurement.
*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p < 0.0001.
NS: Nonsignificant; PI/H: Propidium iodide and Hoechst; RLU: Relative luminescence unit.

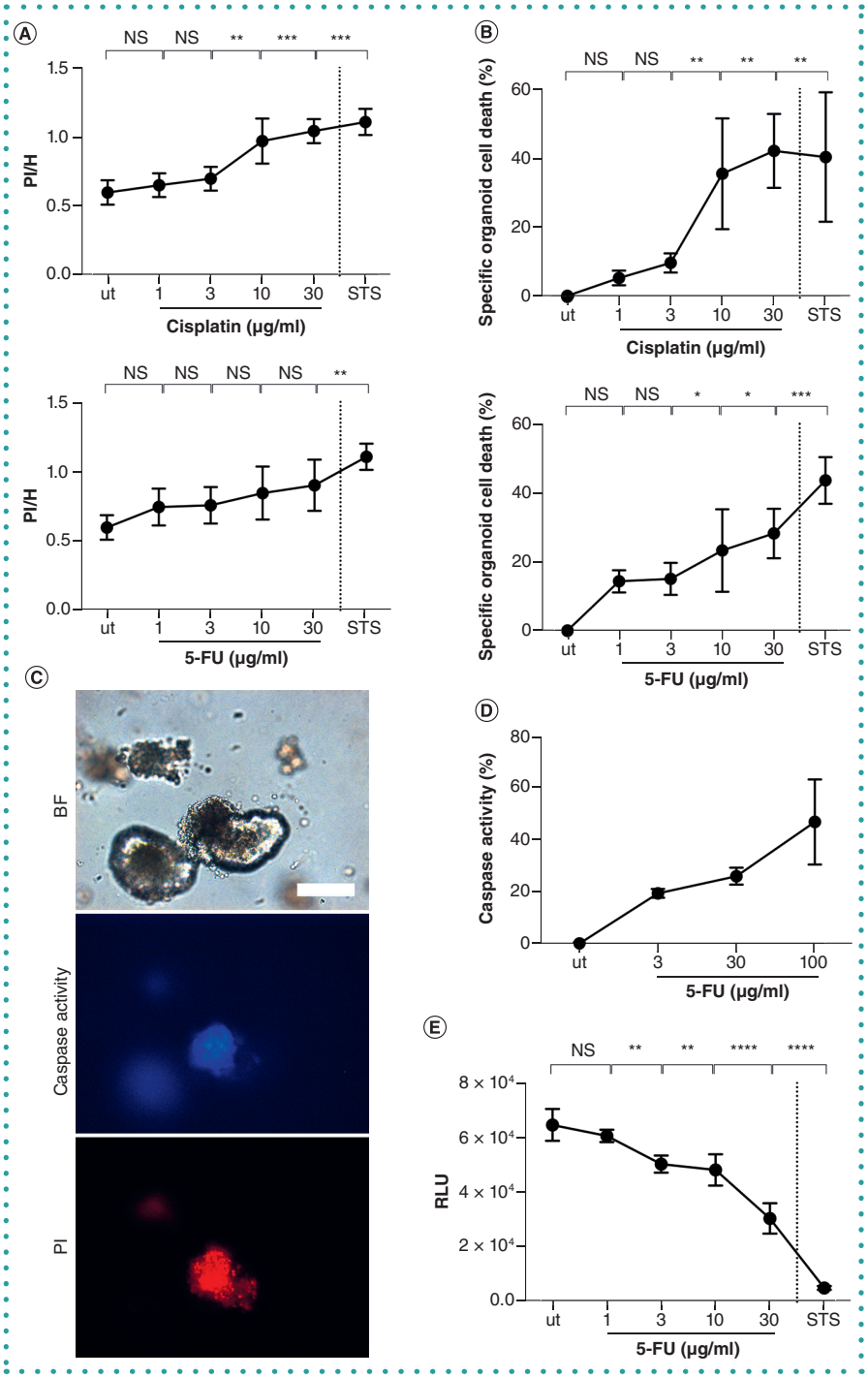

Figure 3. Propidium iodide and Hoechst quantification to calculate specific organoid cell death. Organoids were treated with indicated concentrations of chemotherapeutics and STS (5 µM) overnight. Then, PI/H ratio and treatment-specific organoid cell death were calculated (A & B). (C) Fluorescence image of organoid treated with 10 µg/ml cisplatin overnight and then stained with PI and Caspase 3/7 substrate. (D) Caspase activity was calculated with signal of Caspase 3/7 substrate. (E) RLUs, illustrating intracellular ATP-content, assessed with CellTiterGlo (scale bar = 85 µm). Mean ± standard deviation; n = 3 with technical triplicates.
*p ≤ 0.05; **p ≤ 0.01; ***p ≤ 0.001; ****p < 0.0001.
5-FU: 5-fluorouracil; BF: Brightfield; H: Hoechst; PI: Propidium iodide; RLU: Relative luminescence unit; STS: Staurosporine.

orbital shaker and luminescence was recorded afterwards using a plate reader (Tecan M200 Pro).

Analysis of activated effector caspases

Activation of effector caspases 3 and 7 was performed with CellMeter™ Live Cell Caspase 3/7 Assay Kit (blue fluorescence) according to manufacturer's protocol (AAT Bioquest). In brief, organoids were stained with ApoBrite™ U470 Caspase 3/7 substrate for 2 h at 37°C before treatment. Then, cell death was induced and organoids stained additionally with PI. Subsequently, fluorescence was recorded microscopically (Zeiss Axio Observer.Z1), and quantitatively at 380 nm (ApoBrite) and 617 nm (PI) using a plate reader (Tecan M200 Pro).

Fluorescence microscopy

Intestinal organoids were stained with nuclear dyes as described above and subsequently analyzed in hydrogel (Matrigel/BME) on a Zeiss Axio Observer.Z1 microscope. Brightfield images were taken with Palm-ROBO and fluorescence pictures with AxioVision Software (Zeiss).

Statistical analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Prism Software, Inc.). Unless denoted otherwise, experiments were repeated three times with technical triplicates. One-way ANOVA with Dunnett's multiple comparisons test was performed.

RESULTS & DISCUSSION

Cell death analysis in intestinal organoids in general, and its detection by PI/Hoechst staining in particular, requires precise experimental timing. If organoids are grown for prolonged periods of time dead cells accumulate in the lumen leading to a strong PI background. Therefore, all experiments shown were performed at day 3 after crypt isolation, whereas thawed human organoids were analyzed at day 3 after splitting. Moreover, cell death induction was performed overnight to ensure proper cell membrane disintegration and nuclear staining, independent of the mode of cell death. Initially, organoids were stained for various amounts of time (5–60 min) to assess the optimal duration of staining with both dyes. As stainings with both dyes were

close to completion at 30 min, this time point was used for all subsequent experiments (Supplementary Figure 1).

Figure 1A shows exemplified pictures of murine intestinal organoids treated with the chemotherapeutic drug cisplatin or the pan-kinase inhibitor STS as a positive control. Whereas the PI signal gradually increased with increasing cisplatin concentrations, the Hoechst signal was only slightly attenuated (Figure 1A & B). Quantification of PI and Hoechst fluorescence enabled ratio formation, thus normalizing dying/dead organoids (PI-positive) to total DNA (Hoechst-positive). This ratio is significantly different between untreated organoids and increasing cisplatin concentrations, or STS (Figure 1C). The internal normalization by Hoechst staining stabilized measurements over a wide range of organoid densities (Figure 2B, right), whereas other established methods assessing cellular respiratory potential revealed a strong dependency on cell numbers (Figure 2A & B).

Having established that the ratio of PI/Hoechst signal gradually increased with increasing cell death induced by increasing cisplatin concentrations (Figure 1B), we next aimed at assessing treatment-specific organoid cell death in response to the chemotherapeutic drugs cisplatin and 5-fluorouracil (5-FU) (Figure 3A & B). Increasing concentrations of either chemotherapeutic drug resulted in an increased PI/Hoechst ratio, and an increase in the calculated treatment-specific organoid death (Figure 3A & B). To assess whether cell death observed was associated with apoptosis, caspase activity was analyzed in parallel with PI staining, demonstrating double-positive cells (Figure 3C) and a dose-dependent increase in caspase activity (Figure 3D). Currently, organoid cell death is frequently assessed indirectly by a reduction in intracellular ATP levels [28,29]. The direct comparison revealed that PI/Hoechst staining detects chemotherapy-induced organoid cell death as sensitively as intracellular ATP levels (Figure 3B & E).

In order to verify that cell death analysis by PI/Hoechst staining is not limited to murine primary intestinal organoids, we also assessed cell death quantification with PI/Hoechst staining in murine tumoroids (Figure 4A–C) and human intestinal organoids (Figure 4D), confirming the

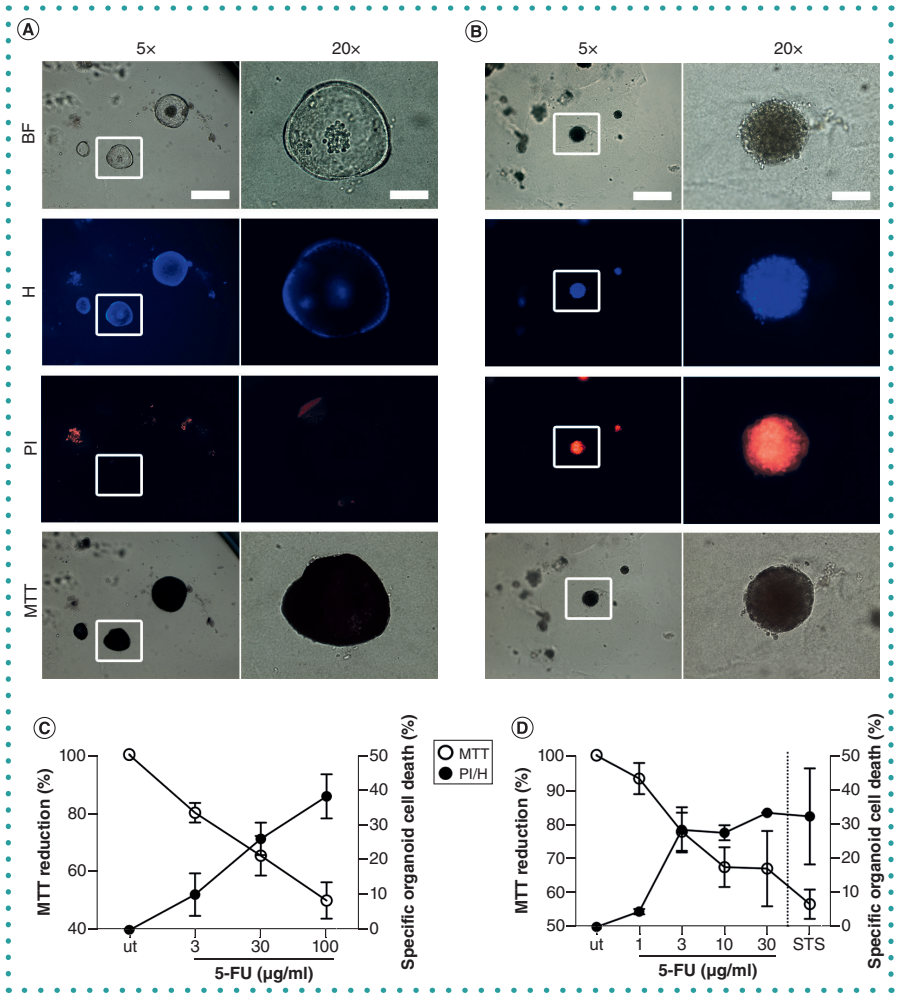

Figure 4. Multiplexing PI/H quantification with MTT-reduction assay. Tumoroids from APC$^{+/-}$ mice were treated with indicated concentrations of 5-FU overnight, stained with PI/H, and fluorescence was quantified. Subsequently, MTT solution was added and MTT reduction was measured. BF and fluorescence microscopic images of untreated tumoroid (A) and 5-FU treated tumoroid (B). (C) PI/H fluorescence derived specific organoid cell death (right axis), MTT reduction (left axis). (D) Human organoids treated with cisplatin. PI/H ratio and MTT reduction were measured, and specific organoid cell death as well as MTT reduction were calculated thereof. Scale bar (5×) = 350 µm, (20×) = 85 µm. Mean ± standard deviation; n = 3 with technical triplicates.
5-FU: 5-fluorouracil; BF: Brightfield; PI/H: Propidium iodide and Hoechst.

suitability of this method for other types of organoids.

We next set to multiplex cell death assessment by combining PI/Hoechst staining with our previously established method of measuring organoid viability by MTT reduction [30]. Therefore, organoids from intestinal tumors of APC^{min} mice (tumoroids) were treated with indicated concentrations of 5-FU, stained with PI and Hoechst, and fluorescence was measured. Subsequently, PI/Hoechst-stained tumoroids were further incubated with MTT and reduction capacity was analyzed by absorbance of the resulting formazan at 562 nm. Whereas viable (ut) tumoroids efficiently reduced MTT to purple formazan (Figure 4A), tumoroids treated with 5-FU showed morphological disintegration and failed to reduce MTT (Figure 4B). A decrease in MTT reduction was paralleled by an increase in PI signal, but stable Hoechst staining (Figure 4A & B). Quantification of PI/Hoechst fluorescence and formazan absorption enabled calculation of treatment-specific organoid cell death (PI/Hoechst), which inversely correlated with decreased MTT reduction (Figure 4C). Multiplexing PI/Hoechst staining and MTT reduction was also confirmed by analyzing cisplatin-induced cell death in human intestinal organoids (Figure 4D). These data ▶

demonstrate that analysis of cell death by PI/Hoechst and cell survival by MTT reduction can be combined in 3D organoid cultures.

In conclusion, we here present a fast and simple method to quantify organoid cell death by measuring PI and Hoechst fluorescence in 3D in a plate reader. The method is cheap, reliable, and does not require commercially available kits, or complicated and time-consuming high content imaging analysis [21,25]. Thus, it is applicable for analysis of treatment-specific organoid death without the need to invest in expensive equipment or bioinformatics. By multiplexing this method with assays assessing respiratory changes, such as MTT reduction [30], information gain is increased and might help to distinguish between direct cell death-inducing agents and inhibitors of cellular respiration and metabolism.

FUTURE PERSPECTIVE

Organoid technology is already being used to investigate patient-specific drug response. Usage of patient-derived organoids is likely toincrease in the future. Thus, fast and robust methods are needed to quantitatively assess cell death in organoids. As our method is fast and simple it can be used frequently in the future to quickly assess death-inducing effects of drugs in PDOs.

SUPPLEMENTARY DATA

To view the supplementary data that accompany this paper please visit the journal website at: www.future-science.com/doi/suppl/10.2144/btn-2019-0023

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$APC^{Min/+}$ mice were a kind gift of Jan Paul Medema, Amsterdam, The Netherlands.

AUTHOR CONTRIBUTIONS

KJB designed the study, conducted most experiments, and wrote the manuscript, SM conducted some experiments and contributed to the manuscript, MS and MM gave technical advice, refined the manuscript and provided human jejunal cells, TB designed and supervised the study, provided funding and finalized the manuscript.

AUTHOR CONTRIBUTIONS

$APC^{Min/+}$ mice were a kind gift from Jan Paul Medema, Amsterdam, The Netherlands.

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No writing assistance was utilized in the production of this manuscript.

ETHICAL CONDUCT OF RESEARCH

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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