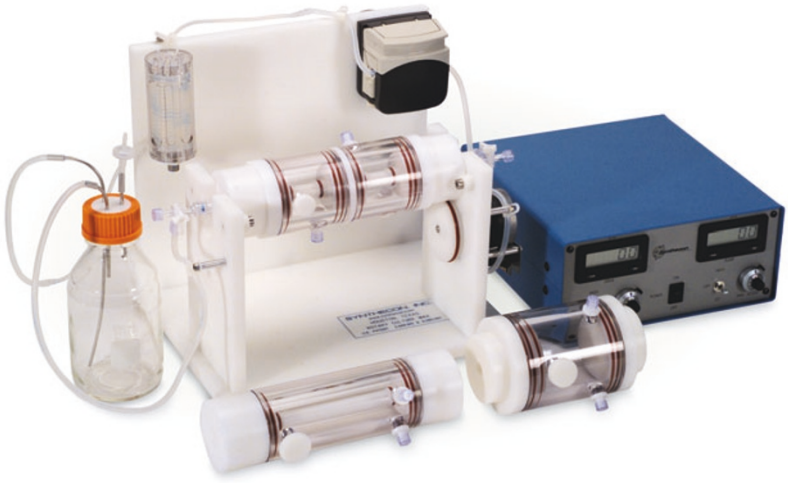
Trouble in the hood: culturing difficult cell types

The culture of animal cells is key to much of basic research today and an important starting point for therapeutic applications. But each cell type has its own quirks. Some cells are happy with most media and protocols, but others can become the bane of a scientist's existence with their seemingly inexplicable needs. Caitlin Smith reports.

"Why some cells start to grow and others do not, we do not know," says Hans Drexler, head of the Human and Animal Cell Line Department at the German Collection of Microorganisms and Cell Cultures (DSMZ). "Some scientists believe in adding various supplements, but my guess is that if cells start to grow autonomously, they grow with or without these supplements—but people, including scientists, are superstitious." Most scientists would balk at the notion of superstition in their protocols, and yet desperate times often call for 'whatever works' measures. This article will focus on some peculiarities and potential remedies of culturing three cell types that challenge researchers today: primary, stem and hybridoma cells.



The RCMW Perfused Flow System showing various culture chamber options. (Courtesy of Synthecon, Inc.)

Primary cells—straight from the source

"In my experience," says Ren-He Xu, senior scientist at the WiCell Research Institute, "most primary cells are particularly difficult to grow. Once established as cell lines, they become much easier [to grow], especially transformed (tumorized) lines. So the difficulty [in growing] them is typically prior to transformation.

The primary culture of animal cells has long been a challenge to cell culturists. Thankfully, there are some media additives that seem to benefit most cultures, such as different types of sera and growth factors. Take human skin cells for example. Yale University School of Medicine's Leonard Milstone, a professor in the Department of Dermatology, says that peptide additives (for example, epidermal growth factor, insulin and basic fibroblast growth factor) are important for culturing both keratinocytes and melanocytes. He uses medium with <0.1 mM calcium to prevent differentiation and prolong proliferative growth. Often one must use the 'try-it-and-see' approach for different additives. Ruth Halaban, a senior research scientist also at Yale's Department of Dermatology, reports that whereas they have little trouble growing cultures of melanoma cells from advanced lesions and metastases, they have difficulties growing melanoma cells from very early primary lesions. The problem, she muses, is that their exact growth factor requirements are simply not known.

Further complicating matters is the exasperating fact that not all cell types respond to additives in the same way. "Very often," says Halaban, "the factors that stimulate the normal cells actually inhibit the malignant cells. We need to perform very meticulous testing to identify the components that can support the proliferation of these primary melanoma cells. This is critical for understanding early changes from normal to malignancy."

Richard Carroll, technical director of the Cell Culture Core in the Center for Molecular Studies in Digestive and Liver Disease at the University of Pennsylvania, reports a similar problem. "One of the problems that we face is the paradox that in a tumor specimen, the untransformed cell contaminants will outgrow the tumor," says Carroll. "Almost all malignant cell cultures grow extremely slowly at first, and often our biggest challenges are preventing normal cell overgrowth and/or contamination." To counteract this effect, Carroll's group grows cultures in the absence of fetal calf serum, which "is not as big a handicap as it might sound," comments Carroll. "Serum often favors the growth of normal cells over malignant cells, compounding the problem of normal cells outgrowing malignant cells."

Some cells in primary culture grow better when provided with so-called 'solid

phase cues', or a three-dimensional (3D) environment that favors cell growth. Carroll sees going from two-dimensional to 3D cultures as a future challenge: "There is some beautiful work out there showing that any number of basic cellular properties are altered when the cells are cultured in three versus two dimensions." While it is impossible to know the exact matrix conditions *in vivo*, many companies offer matrix or chamber products that claim to better replicate the 3D *in vivo* environment—for example, Chemicon's 3D Cell Culture Kit, 3DM's Puramatrix, and Oligene's Perfusion Chamber System PCS 3c. Bill Anderson, president and CEO of Synthecon, agrees that "the importance of '3D biology' [has] only recently [started to be] fully understood and appreciated." Synthecon offers the National Aeronautics and Space Administration (NASA)-designed Rotary Cell Culture System, a bioreactor capable of growing 3D cultures.

Manufacturing of Cell Culture Technologies' media at B. Braun Medical AG, Crissier/Lausanne, Switzerland. (Courtesy of Cell Culture Technologies, LLC.)

Stem cells—creating new cell types

As a type of primary cell, stem cells are in a category all their own. Whereas their pluripotency has earned them fame and controversy, there is a long way to go before their enormous therapeutic potential is realized.

Once the stem cells of interest are isolated, they can be quite difficult to establish in culture and to grow thereafter. According to David Schaffer, assistant professor in the department of chemical engineering

and the Helen Wills Neuroscience Institute at the University of California Berkeley: "Sometimes they simply die after isolation, and one must do a protocol optimization to overcome this if it presents too much of a problem. Since isolation protocols are lengthy, the optimization would be as well."

Once cultures are established, scientists growing human embryonic stem (ES) cells need to prevent stock cultures from differentiating into the over 200 cell types that they can become. Typically ES cells require a medium supplemented with serum and are grown on a feeder layer of mouse fibroblasts, which helps to keep the stem cells in their undifferentiated state. This arrangement is less than ideal for clinical research purposes, because the presence of animal contaminants in the medium and the feeder layer can make it unsafe for future therapeutic applications in humans. To alleviate the problem, the feeder cell layer is often replaced by a mouse-derived cell matrix extract (such as Matrigel from BD Biosciences) in combination with conditioned medium from a mouse fibroblast culture. Similarly, the animal serum can be replaced by a serum-replacement formulation (such as Knockout Serum Replacement from Invitrogen). But these tricks do not solve the problem of animal contaminants. According to Schaffer, a substantial challenge in the near future will be "the development of serum-, feeder- and protein substrate–free cultures for growing stem and other cell types."

An important step in this direction was taken in March when two independent groups reported that a specific formulation of growth factors can block differentiation and replace mouse fibroblast conditioned media for the culture of human ES cells[1,2]. Whereas this discovery will go a long way toward making the use of ES cell cultures safer for clinical use, it does not mean that the culture media are yet free of animal components: a bovine-derived serum replacement and a mouse-derived matrix gel are still required.

After successfully isolating and maintaining your stem cell of interest, you may want to induce differentiation. R&D Systems offers kits to facilitate the differentiation of ES cells into dopaminergic neurons and oligodendrocytes under serum-free conditions. The kits contain supplements to enrich neural stem cell populations; bovine fibronectin as a matrix for cell attachment and spreading; and the basic human fibroblast growth factor, mouse fibroblast growth factor 8b and mouse sonic hedgehog amino-terminal peptide (for dopaminergic neurons), or human epidermal growth factor and human platelet-derived growth factor AA (for oligodendrocytes). R&D Systems estimates that the kit contents are sufficient for the differentiation of 3×10^7 ES cells.

Hybridoma cells—antibodies galore

Monoclonal antibodies, whose value in basic research is undisputed, are produced from hybridomas, immortalized cell hybrids resulting from the fusion of spleen cells from an immunized mouse with a continuous myeloma cell line. Hybridomas are tra-

BOX 1: *MYCOPLASMA*: EVERY CELL CULTURIST'S NIGHTMARE

Mycoplasma—the name alone can evoke dread. This is the genus name of a prokaryotic organism so small (approximately 0.15–2 μm in diameter) that it passes through sterilizing filters. Conventional cell culture antibiotics don't work because these agents target cell walls, which *Mycoplasma* lack. They are sneaky too, living in cultures without overgrowing the cells, so you may not know they're present unless you test frequently. And test you must—*Mycoplasma* can affect virtually every normal cellular process, wreaking havoc on data collection and requiring the repetition of many experiments.

The many testing kits available work in different ways, using methods such as PCR, biochemical assays, enzyme-linked immunoabsorbent assay (ELISA) and immunofluorescence. Richard Carroll, technical director of the Cell Culture Core at the Center for Molecular Studies in Digestive and Liver Disease at the University of Pennsylvania, reports success with the MycoAlert Mycoplasma Detection Assay from Cambrex. This biochemical test relies on the activity of mycoplasmal enzymes to react with the MycoAlert substrate, catalyzing the conversion of ADP to ATP. Measurement of ATP before and after the reaction indicates whether *Mycoplasma* are present. Cambrex claims that this test is simple, quick (less than 20 minutes) and reliable even with low levels of contamination (capable of detecting *Mycoplasma* present at less than 50 colony forming units per milliliter).

The PCR-based tests use primers to amplify the 16S and/or 23S ribosomal RNA genes of *Mycoplasma*—a positive result is indicated by the presence on a gel of a band from the PCR product. Sigma-Aldrich's VenorGeM Mycoplasma PCR Detection Kit uses this principle and takes about four hours. Stratagene's new MycoSensor QPCR Assay Kit detects *Mycoplasma* using real-time quantitative PCR in about two hours, and includes internal controls to reduce false positives and false negatives.

If you want to avoid using PCR, you might try R&D Systems' MycoProbe Mycoplasma Detection Kit. This system uses labeled oligonucleotide probes that hybridize to the 16S ribosomal RNA of the eight most common *Mycoplasma*. Detection is facilitated by a probe labeled with alkaline phosphatase and a colored substrate solution. R&D Systems claims that this test takes approximately 4.5 hours.

Finally, if you don't want to do the testing yourself, you can outsource the job by sending your culture samples to a company that offers *Mycoplasma* testing services, such as Baseclear, Bionique Testing Laboratories, Cell Essentials and Mycoplasma Experience.

So you've tested and found a positive culture? Alas, usually the best strategy for dealing with an infected culture is simply to throw it away and begin anew. But what if your culture is unique, and has no uninfected backup? Some have had success treating cultures with the antibiotic plasmocin, which targets *Mycoplasma* protein synthesis and DNA replication. Plasmocin is available from InvivoGen and Cayla, among others. Sigma-Aldrich also offers its nonantibiotic Mynox Mycoplasma Elimination Reagent.

ditionally grown in medium supplemented with bovine serum, but as with stem cells, a recent challenge has been the need to grow them in serum-free medium with no animal proteins. The issue arises from the fact that monoclonal antibodies are increasingly useful as human therapeutic agents.

Manufacturers are beginning to fill this need for serum-free medium. According to Mark Hirschel, CSO of Biovest and director of the National Cell Culture Center, "the most important improvement we've seen is the development of better commercially available serum-free media." But he believes that a challenge remains to develop "robust animal component–free medium that is equivalent to serum-containing medium. Serum is expensive, so animal component–free medium needs to be less expensive yet be suitable for several cell types."

For example, Cell Culture Technologies offers their TurboDoma media for culturing myeloma and hybridoma cell lines, as well as kits aimed at saving researchers' time. Ferruccio Messi, the president and CEO of Cell Culture Technologies, says

BOX 2: CELL CULTURE FROM A DISTANCE

Perhaps you're tired of the painstaking daily maintenance that successful cultures demand. Maybe you'd like to free up your scientists to work on other projects. A number of companies and organizations provide contract cell culture services that may meet your outsourcing needs. Their expertise could make all the difference, especially if you need to change to culture protocols or use unfamiliar techniques.

For example, workers at Cell Culture Solutions have experience with a range of protocols and technologies in the culture of 'industrial' cell lines (such as hybridomas), as well as human primary cells used for assay development and diagnostics. 'Most of our requests are for process development, such as bench scale up, assay development and cell culture performance tests," says Brigitte Van der Haegen, Cell Culture Solutions' managing director. 'These requests include laboratory and/or consulting services. We also have several requests for cell expansion." Cell Culture Solutions uses both traditional and newer culture methods, says Van der Haegen, citing the use of 'suspension and microcarrier cultures for industrial and scale up applications. We also like to use permeable bags and disposable bioreactors whenever scale-up work is required." According to Van der Haegen, her company has experience with both cell lines and primary culture: 'Therefore, we can offer services to those working with industrial cell culture (production of biologicals) and to those working with human cells (organ transplantation, drug testing, tissue engineering)."

You may also want to consider the National Cell Culture Center (NCCC) for your contract cell culture work. The NCCC was established by the National Institutes of Health (NIH) for the purpose of providing customized, large-scale cell culture services for basic research labs. The NCCC claims experience with over 600 cell lines, including common lines routinely produced by NCCC such as HeLa cells, CHO cells, K562 cells, HEK 293 cells, Sf9 cells and hybridomas. If you want to scale-up your work, the workers at NCCC can start with your protocol, then adapt it to scale-up, and deliver the cells to you for a nominal fee. The NCCC is funded mainly through the NIH to minimize costs. Anyone conducting basic research may use the NCCC services; fees cover materials as well as some labor and operational costs.

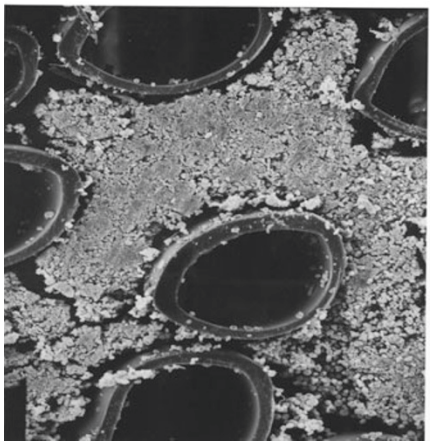
If you choose to send your cell line to the NCCC, it will be quarantined until shown to be free of *Mycoplasma* and other contaminants, and samples will be stored in their cell bank. Or you may choose to use one of the more common cell lines already in routine use at the NCCC. Soon after, the NCCC will send you a cell sample, a production schedule and a price quote. The large-scale production services offered include mammalian cell culture (1 to 400 liters per day for suspension or 1 to 200 roller bottles per batch for anchorage-dependent cells), insect cell culture using baculovirus (1 to 50 liters per day), and purification of monoclonal antibodies (10 mg to 100 g) or nonhybridoma cell-secreted proteins.

Other commercial companies also provide contract cell culture services, including Cell Essentials, Cell Trends and Paragon Bioservices.

"we offer chemically defined, protein- and peptide-free minimal culture media for growing well-established animal cell lines. All our media are made of pharmaceutical-grade small molecules of nonanimal origin. We do not use complex additives such as hydrolysates, yeast extracts, albumins or proteins, not even insulin, to produce our culture media."

For scientists wanting a quick start to serum-free culture, Cell Culture Technologies also offers Starter Kits containing a hybridoma cell line grown in the absence of animal proteins, in minimal culture medium free of proteins and peptides, and protocols from the European Collection of Cell Cultures for maintenance and banking of the cell line. "Today, too many scientists in academia and industry waste too much time selecting cells for serum-free culture," says Messi, "so we thought that providing the essential tools at once might help scientists to concentrate on their actual research targets instead of wasting time with boring selection procedures."

Maximizing antibody production is an important goal in growing hybridomas, and the culture vessel may have an effect on cell growth and monoclonal antibody output. FiberCell Systems claims that hybridomas grow exceptionally well in their proprietary hollow fiber cells. FiberCell Systems' president John Cadwell explains that "because of the tremendous amount of surface area offered in such a small volume, and the high gross filtration rate of our proprietary fiber, cells will grow at extremely high densities. This permits easy adaptation to serum-free medium or the reduction of serum to as low as 2% of the total volume of the medium with no problems." Another advantage to their system is that their fiber allows transforming growth factor β, a secreted factor inhibitory to hybridoma growth, to diffuse away while retaining the antibodies in the small volume of the extracellular space. According to Cadwell, hybridomas grown in their fiber cell systems can produce up to two grams of monoclonal antibody per month.



Cross-section through hollow fiber culture system showing extracapillary space. (Courtesy of FiberCell Systems, Inc.)

Thinking about the future

Whereas great strides have been made in cell culture technology, there remain even greater challenges to the field in the near future. For example, the importance of tracking karyotypic changes has

received too little attention, according to Xu. "Cultured cells are continuously under selection pressure," he stresses. "Only those with selection advantages will survive and expand. To track and maintain the karyotypes of each specific cell type is vital for the usefulness of the cells in research."

In the culture of primary cells, Halaban says that continuing to identify growth factors that stimulate proliferation and differentiation is an important technical challenge. In addition, she suggests the creation of government-supported centers of excellence that distribute cells to researchers for a nominal fee. "Growing human cells is very critical for continuing research," remarks Halaban. "It takes specialized skills that cannot be developed by every scientist."

Indeed, Brigitte Van der Haegen, managing director of Cell Culture Solutions, agrees that cell culture is transitioning from being merely "a supporting technique to a biotechnology." Despite the current development of new tools in cell culture, she believes that the key to optimizing cell culture is rooted in cell biology. Van der Haegen remarks that scientists "involved in the optimization of cell culture systems (irrespective of their application) need to focus on understanding the biology of cells in order to achieve their goal."

FiberCell's hollow fiber cell culture system. (Courtesy of FiberCell Systems, Inc.)

Other organizations and commercial companies provide private cell banking services, including the National Cell Culture Center and Cell Essentials.

1. Xu, R.-H. *et al. Nat. Methods* **2**, 185–190 (2005).
2. Xu, C. *et al. Stem Cells* **23**, 315–323 (2005).

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BOX 3: SAFEKEEPING: CELL LINE REPOSITORIES

Just when you think you've perfected your cell lines, a power failure warms your lab freezer and ruins your stored culture lines. Why, you think, didn't I keep some cells in a safer place? You might also consider submitting your perfected cell line to a bank, so that other scientists can build upon your work. Several organizations provide repositories to serve the noble purpose of sharing cell lines, and the useful purpose of safekeeping.

The American Type Culture Collection (ATCC), the European Collection of Cell Cultures (ECACC) and the German Collection of Microorganisms and Cell Cultures (DSMZ) are nonprofit organizations that act as repositories for cell lines. They accept submission of cell lines only after consultation with staff scientists to evaluate whether the cell line would be a valuable addition to their repositories. Submitting a cell line is free, and the donor receives several advantages. The cell line is subjected to a battery of tests, including DNA profiling and *Mycoplasma* testing. The cell line may be further characterized, possibly yielding additional information about the cells, and is also safely cryopreserved for future use. The donors no longer need store the cell line or subculture it for distribution to others. And there is also the good feeling that you have contributed to the larger scientific community, allowing others to benefit from your success. Additionally, the ATCC, ECACC and DSMZ provide safe deposit services and patent deposit services, for a fee. With these services, you are the only one who has access to your deposited cell line.

SUPPLIERS GUIDE: COMPANIES OFFERING CELL CULTURE PRODUCTS AND SERVICES

Company	Web address
American Type Culture Collection	www.atcc.org
Baseclear Labservices	www.baseclear.com
BD Biosciences	www.bdbiosciences.com
Bio Express Cell Culture Services	www.bio-express.com
Bionique Testing Laboratories	www.bionique.com
BioVest International	www.biovest.com
Cambrex	www.cambrex.com
Cascade Biologics	www.cascadebio.com
Cayla	www.cayla.com
Celartia	www.celartia.com
Cell Culture Technologies	www.cellculture.com
Cell Culture Solutions	www.cellculturesolutions.com
Cell Essentials	www.cell-essentials.com
Cell Trends	www.celltrends.com
Cellutron	www.cellutron.com
Chemicon	www.chemicon.com
Clontech	www.clontech.com
German Collection of Microorganisms and Cell Cultures	www.dsmz.de
European Collection of Cell Cultures	www.ecacc.org.uk
Endogen	www.endogen.com
Fiber Cell Systems	www.fibercellsystems.com
Fisher Scientific	www.fishersci.com
Genovac	www.genovac.com
Green Mountain Antibodies	www.greenmoab.com
Hyclone	www.hyclone.com
INCELL	www.incell.com
Invitrogen	www.invitrogen.com
InvivoGen	www.invivogen.com
JRH Biosciences	www.jrhbio.com
Maxim Biotech	www.maximbio.com
Metachem Diagnostics	www.metachem.co.uk
Millipore	www.millipore.com
Minerva Biolabs	www.minerva-biolabs.com
Minucells and Minutissue Vertriebs	www.minucells.de
Mycoplasma Experience	www.mycoplasma-exp.com
National Cell Culture Center	www.nccc.com
Nexcelom Bioscience	www.cellometer.com
Nunc	www.nuncbrand.com
Oligene	www.oligene.com
Paragon Bioservices	www.paragonbioservices.com
PromoCell	www.PromoCell.com
3DM	www.puramatrix.com
R&D Systems	www.RnDSystems.com
Roche Applied Science Writer	www.roche-applied-science.com
Sigma-Aldrich	www.sigmaaldrich.com
Specialty Media	www.specialtymedia.com
StemCell Technologies	www.stemcell.com
Stratagene	www.Stratagene.com
Synthecon	www.synthecon.com
Wave Biotech	www.wavebiotech.com
WiCell Research Institute	www.wicell.org