

Tips and Techniques for Enhancing Your Cell Culture

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Abstract

Many advances in the understanding of the behavior and function of biological systems are dependant on the study of cells and tissues. An essential tool in this research is the use of *in vitro* cell culture.

Many variables contribute to providing a physiological environment for the cell in the laboratory, a few examples include cell source, isolation techniques, growth conditions such as matrix proteins and soluble factors, and cell age. Basic laboratory practices are sometimes overlooked as a source of discrepancy in data; however the application of fastidious and reproducible technique can reduce cell culture as a source of data variation. As specialized techniques have been developed to modulate cells and tissues *in vitro* the importance of reproducible data has become paramount.

In this presentation we will identify and discuss basic principles of *in vitro* mammalian cell culture that influence the quality of experimental results.



Why Culture Living Cells?

To Study:

- Intracellular activities
 - DNA replication
 - Transcription (RNA)
 - Translation (protein)
 - Signal Transduction
- Cell-environment interaction
 - Drug action, infection, cytotoxicity, receptor/ligand interaction
- Cell-cell interaction
- Genomics and proteomics

To Produce:

- Cells, cell products, and cell secreted products
 - Protein, DNA, RNA, antibody, transfected protein product



Slide 1

I'd like to start by welcoming you to the webinar, and thanking you for giving me the opportunity to speak about some tips and techniques that you can introduce into your cell culture labs. At the end of the webinar is a slide that will give you the contact information for our technical support group. They are available by phone or e-mail, and can help you with questions and comments. Also on the slide will be my phone number and e-mail address, if you'd like to contact me directly with questions or comments.

Slide 2

I am an R&D scientist based in Bedford, Massachusetts. Our lab is primarily focused on phenotypic modulation of cells, by controlling the *in vitro* cell environment, including surface substrates such as extracellular matrix proteins, non-biological modifications of these surfaces, and soluble factors in media. We also develop cell-based assays such as those used in angiogenesis studies, tumor biology, and stem cells. The *in vitro* culture of cells is a very important tool in our laboratory. We have found that being able to standardize techniques used by individual contributors in the lab reduces the data variability of our experiments, and by that, the quality of our work. We'll start with a few introductory slides, followed by a discussion of a series of factors that can influence your experimental data. Many of these could be entire webinars – some factors we won't even discuss. Today is simply to highlight some of the common areas where research has introduced variation into data.

Slide 3

So why culture living cells? We primarily do that to do mechanistic studies such as intracellular activity, cell environment interactions, and cell-to-cell interactions, as well as the production of viruses, protein products of cells, and in an emerging area, the cells themselves for clinical cell therapies.



Why Standardize Techniques?

- Cultivation in a more physiological environment
= **increased value of experimental data**
- Cultivation in a controlled environment
= **defined parameters that can be changed/measured**
- Cultivation in an optimized environment
= **increased signal/noise ratio**



Why Standardize Techniques?

As an example:

It is estimated that it takes ~11 years and ~\$800 million to develop a new drug

- Tufts Center for Study of Drug Development
<http://csdd.tufts.edu/>

Failure of Drug Candidate is 39% when moving from ADME to Clinical Testing

- Price Waterhouse Coopers (1999)

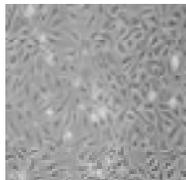
A 10% improvement in predicting compound failure before clinical trial could save up to \$100 million in development costs per drug



Experimental Data

Experimental Data is Influenced by:

- Cell Source
- Initial Growth Conditions
- Medium Composition
- Cell Counting Procedure
- Physiological Parameters
- Characterization
 - Age, Karyotype
- Substrate, Matrix, or Not
 - Morphology
- Dissociation Method
- Contamination Control
 - Aseptic Technique
- Cryopreservation



BD™ Human Umbilical Vein Endothelial Cells, Cat. No. 354151



Slide 4

Why should you use standardized techniques? A good physiological environment is key to good data. As a researcher, you need to understand what behavior you are trying to study, and then provide the cell with the appropriate environment *in vitro* to mimic that behavior. Controlling this environment allows you to change and measure experimental data. An optimized culture environment also allows a cleaner measurement of signal-to-noise ratio.

Slide 5

I'd like to use pharmaceutical research and development as an example that can be expressed in monetary terms. The Tufts Center for the Study of Drug Development estimates that it takes eleven years and \$800 million to develop a new drug. If we could improve prediction of compound failure by 10%, the resulting savings would be in the \$100 million range per drug. Most of our cell culture work does not occur under these circumstances. However, you can imagine how big the impact of poor data quality would be on your work.

Slide 6

Today's webinar will highlight some of the key areas that influence experimental data. In each of these ten areas listed on this slide, we will give you examples of how data is affected by the lack of standardization. We will also direct you to literature sources that can help to expand upon this information, and allow you to implement these changes into your lab.

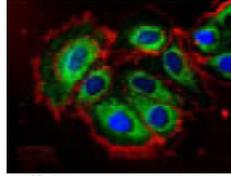


Cell Source

Primary Cells:

Cells isolated from an organism prior to the first subcultivation

- Tissue Source
- Isolation Technique
- Species
- Donor



MCF-7 (breast cancer cell line) labeled with Hoechst (blue), MitoTracker green (green) and wheat germ agglutinin (red)
BD Pathway™ 855 Bioimager

Cell Line:

Propagated culture after the first subculture

- Certified Stock
- Not your neighbor

Cross-Contamination of Cell Cultures:
A Call for Vigilance and Authentication

Journal of Cellular Biochemistry

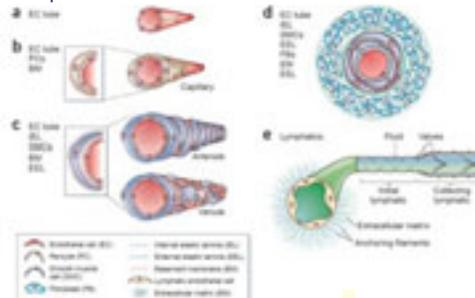


Slide 7

We'll start with cell source. This is the first line of defense in standardizing a cell biology laboratory. A cell line is a culture that has been sub-cultured from a primary isolation. It is important to use cells that have clear lineage, cells that you have had under your control, and that you understand. This is done by beginning with an authenticated seed stock. There are many cell repositories. Some examples are the American Type Culture Collection, and the Cornell Institute for Medical Research in the U.S. In the U – E.U., European Culture for Cell Cultures, and the German Collection of Microorganisms and Cell Cultures, and in Japan, the Riken Bioresource Center. It is important to avoid borrowing cells from the lab down the hall. It's been estimated that 15% to 19% of published cell culture based research uses cells that are misidentified or cross-contaminated. I would direct you to the July 2007 newsletter of the *American Society of Cell Biology*, where this issue is highlighted. There is a reference list included in this article. Primary cells are cells that are isolated directly from an organism prior to the first subcultivation. These cells have unique issues that I would like to go through in more detail. They are: tissue source, isolation technique, species, and donor.

Cell Source: Tissue Source

Example: Endothelial Cells

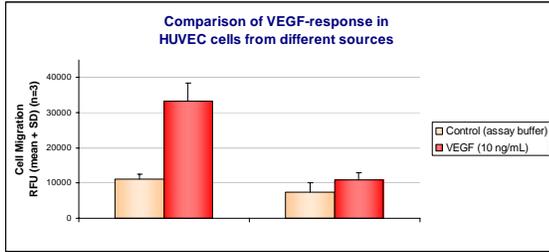


Slide 8

As an example of variability due to tissue source, I like to use endothelial cells. Endothelial cells come from many sources throughout the body, and also they may all express endothelial markers, they are not all alike. There are differences in large vessel versus endothelial cells from microvascular sources, each cell having unique properties. Endothelial cells from arteries and veins have both anatomical and physiological distinctions – the blood pressure that they sustain, and in the thickness of smooth muscle cell support around them. Protein sets that they express can be different. For example, the expression and activity of angiogenic factors such as VEGF, and in a later slide, I'll show you an example of this. It is important for you, as a researcher, to understand what you are studying, and be sure that the cells that you use are appropriate for your intent.



Cell Source: Donor Variability



*The data in this graph is representative of 3 independent endothelial cell migration experiments.

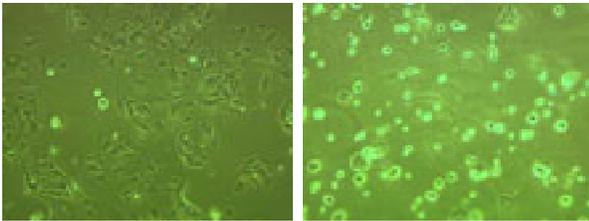


Slide 11

The final cell source watch out is donor variability. This example is of human umbilical vein endothelial cells from two sources. The experiment is cell migration through a microporous membrane based support, commonly called a cell culture insert, towards the angiogenic growth factor VEGF, as compared to a control which contains only assay buffer. The light-colored bar is assay buffer – the dark bar is migration towards 10 ng/mL of VEGF. You can see that the two sources vary in response. On the left, a nice migration response to VEGF from cell donor A – on the right, no response from donor B. Donor to donor variability is a common source of data variability. This is an important parameter to evaluate in drawing conclusions from your data.

Initial Growth Conditions

Example: Caco-2 Cell Culture



Collagen 1

TC-Treated Polystyrene



Slide 12

Using Caco-2 cells again, I'd like to talk about initial growth conditions. On the left are cells from a cryopreserved vial at 24 hours, cultured on BD BioCoat™ Collagen I on the right, grown on TC treated polystyrene plastic, both using the same cells, same media. Collagen I demonstrates better attachment during those first 24 hours as the cells proceed through logarithmic growth. The result, more cells faster. Most Caco-2 users know to allow the cells a few days to attach before a medium change. The next slide, however, demonstrates how important it is to understand what it is doing to your cells.

Initial Growth Conditions

Example: Caco2 Cell Culture

Digoxin Permeability Comparison

Caco-2 Line	Pore Size	Papp		Papp Ratio	
		A-B	B-A		
10% FBS	1 uM	mean:	2.9	9.7	3.6
		SD:	0.8	1.8	
		%CV:	27	19	
		(n=4)			
20% FBS	1 uM	mean:	1.3	17.3	14
		SD:	0.24	2.50	
		%CV:	19	14	
		(n=8)			



Slide 13

This data has been generated in our contract research group. Digoxin is a control standard that we routinely use in our permeability assays. It is a commonly used drug, has good human bioavailability, and is estimated to be absorbed at about 66% to 90%. It is absorbed by transcellular diffusion, and subject to the multidrug resistance 1, MDR1 gene product, the G-glycoprotein PGP efflux. We use this compound to test our technique for culturing Caco-2 cells, and found that when we used 10% FBS during the initial growth phase of the cells, we selected out for cells that did not express PGP. This is demonstrated by the lower apparent permeability of the cells grown using media containing 10% fetal bovine serum. A smaller apparent permeability Papp ratio indicates less expression of PGP. The PGP ratio of 14, when using 20% fetal bovine serum, demonstrates the expected transport of this compound. Since we want Caco-2 cells to demonstrate and test for transporters, then the higher expressing cells are the preferred cells. Using 20% fetal bovine serum gave us this preferred result. This example demonstrates how important it is to understand and control the environment that you expose yourselves to, and that you control for this environment before and during your experiments.



Medium Composition

Must supply proper nutrients, including essential amino acids, pH balance, proper osmolarity, proper surface tension and viscosity, and buffering for gas exchange

- Serum supplies a variety of required components
 - Growth factors and hormones
 - Proteins, including those needed for adherence
 - Trace minerals
 - Inhibitors of cell proliferation
 - Cell proliferation inhibitors can enhance differentiation



Media Watch-outs

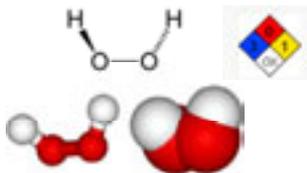
- Control for water source and reagent preparation
- FBS
 - Lot-to-lot (uncontrolled) variation in hormone and growth factor levels
 - Test FBS or use serum-free
- Antibiotics or Not
- Feed cells every 2 days, warm media to 37°C
- Store media at 4°C *in the dark*, observe expiration dates



Photo-decomposition of Medium Components



Fluorescent Lighting
 HEPES, Riboflavin, Tryptophane
 ↓
 Hydrogen Peroxide and Free radicals
 =
 Toxic to Cultured Cells



Slide 14

Media composition can be an entire training class. The liquid environment that is supplied to your cells must provide the proper nutrients, pH balance, osmolarity, surface tension, and viscosity. It must also be capable of buffering for gas exchange. Serum can be the source of growth factors and hormones, proteins – including those needed for cell adherence, trace minerals, and it can also be the source of inhibitors for cell proliferation, and aid in the differentiation of cells. Serum free medium has become an important area of focus, in order to remove sources of undefined components to the media.

Slide 15

Some media watch outs are to control for water source and reagent preparation. If you are making media from powder, your water and reagent preparation are key components to the environment that your cells see. The quality of the water is contributing to the behavior of your cells, and should be closely controlled. Fetal bovine serum has lot-to-lot uncontrolled variation in hormone and growth factor levels. You can control for this by testing new lots of fetal bovine serum as compared to your current lot, or move towards the use of serum free media. Antibiotics or not? Every tissue culture lab must answer this question. In our lab, we try to avoid the use of antibiotics, as they mask suboptimal aseptic technique. We do, however, use antibiotics in primary cultures, as it is very difficult to isolate primary cells in a sterile manner. The next bullet point is there as a placeholder. This is a standard method – feed cells every two days, warm media to 37 degrees. However, every cell type has different requirements. This should be evaluated, and a protocol set for each type. You always want to store your media at four degrees, in the dark, and observe expiration dates.

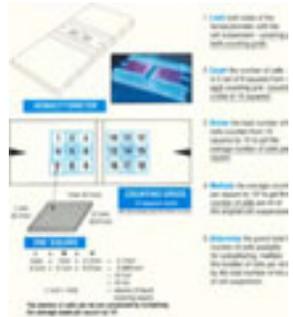
Slide 16

This becomes important, because fluorescent lighting can photo-decompose some media components such as riboflavin and tryptophane into hydrogen peroxide and free radicals, both of which are toxic to cultured cells.



Cell Counts

- Standardized technique
- Cell preparation
 - Single cell suspension
 - Dilutions
- Sampling technique
 - Well mixed
 - Accurate pipetting
- Filling of chamber
 - Over/under filling
 - Bubbles
- Clean chamber, even distribution
- Coverslip, heavier than normal
 - Coverslip to maintain
 - Chamber height



Slide 17

I would like to move on to another area where variability is introduced into experiments, and that is in cell counting techniques. On the right side of the slide is a BD reference sheet for the proper use of a hemacytometer. But I'd like to draw your attention to the left side of the slide. First and foremost, standardized technique. Everyone in your lab should be using the same technique to count cells. We find that many issues in cell-based assays come from inconsistency in cell counts from one researcher to another. Cell preparation is also very important. Cells should be in a single cell suspension. Adding clumpy cells to a hemacytometer does not allow for even loading. The gap between the hemacytometer and the coverslip is 100 microns. Large clumps of cells will be excluded from the load volume, and cause uneven distribution of the suspension under the glass. Make sure that your sampling technique is accurate. Use well-mixed populations of cells, accurate pipetting, and even filling of the chamber. Be sure that there are no bubbles, and that you do not over or under fill the slide. In order to get even distribution of the cells, the chamber must be clean. Many times, a previous user may not have cleaned the chamber, and you will find areas of dried protein on the glass. This will cause uneven distribution of cells. Our lab has had many issues concerning the hemacytometer coverslip. The coverslip drops on the floor, gets broken, and a substitute coverslip is used from whatever is handy in the lab. Hemacytometer coverslips are heavier than normal coverslips, in order to maintain chamber height, which is critical to the control of the volume in the chamber, and in getting an accurate count. Be sure to add dilutions to your calculations.

Physiological Parameters

- Temperature: 37-38.5°C
- pH: 7.2-7.4
- CO₂ @ 5% in 95% air
- Oxygen Tension: 1-10%
- Osmotically Balanced Medium
- Humidification: saturated
 - Limit evaporation to maintain osmolarity



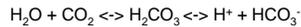
Slide 18

The next few slides concern physiological parameters important to the cells – for example, temperature, pH, and CO₂. I have included a few photos from our lab so that I could point out that we calibrate our incubators to standards traceable to the National Institute of Standards and Technology – NIST, a federal technology agency that develops and promotes measurement standards and technology. We use a daily check chart to avoid the Monday morning realization that the CO₂ tank ran out over the weekend. Cell health is determined by morphology, growth rate, plating efficiency, and expression of function. Typically, cultures of warm-bodied animals are grown at 37 degrees – cold blooded animal derived cells at temperatures that are generally near the upper limit of optimal body temperature for the intact animal. Mammalian cells are generally most happy at 36 to 37 degrees. Osmotic pressure regulates the flow of substrates in and out of the cell, so avoid evaporation of the media at all costs. DMEM is a bicarbonate-based media, and is used for CO₂ regulation at 7.2 to 7.4. There are alternatives such as organic buffer HEPES, which can be used to supplement bicarbonate-based media, and regulate pH independent of the CO₂ concentration.



Physiological Parameters: pH

- Most Mammalian Cells grow well at pH 7.4
- Cells produce waste products that tend to be acidic
- Thus, generally require a buffered medium
- Sodium bicarbonate is usually the buffering agent
- The pH is governed by the reaction



- Cultures must be incubated in a CO₂ environment in equilibrium with the H₂CO₃ in the medium

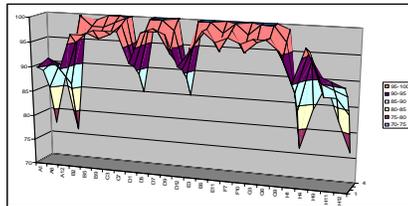


Slide 19

Most mammalian cells grow well at pH 7.4, but cells produce waste products that tend to be acidic, secreting these waste products into the media, and acidifying the media. This is generally handled by using a buffered media, usually sodium bicarbonate, and the pH is governed by the reaction on this slide, which you can see in the middle of the slide. This is influenced by both the concentration of sodium bicarb, and also in the CO₂ concentration of the gases in your incubator. Cultures must be incubated in a CO₂ environment in equilibrium with the sodium bicarbonate in the media. In the upper right hand corner of the slide is a picture of vented caps. Make sure that your culture chamber allows for a free exchange of gases from the incubator to the cell culture media.

Physiological Parameters: Humidity

- 96-well plate evaporation study
- Plate map of volumetric changes over time



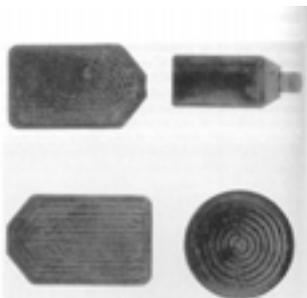
B.K. Lundholt, et al., A Simple Technique for Reducing Edge Effect in Cell-Based Assays. J. Biomolecular Screening 8(5), 566-570, 2003.



Slide 20

In order to maintain equilibrium between the culture media and the gaseous carbon dioxide/air mixture in the culture chamber, dishes cannot be sealed. In order to avoid evaporation of the media and subsequent changes in osmolarity, the humidity of the incubator must be as close to saturation as possible. This data is an example of what a plate map, measuring volumetric changes over four days, can look like when there is evaporation of the media. Wells at the outer edge of the plate lose volume first. In our lab, we use stainless steel dishes at the bottom of the incubator. These are filled daily to avoid the evaporation of the media from the wells. I have also included at the bottom of the slide a reference that can help you evaluate methods to reduce the edge effect in cell-based assays.

Vibration



Non-random adherence and growth of cell suspension due to vibration or resonance in the incubator



Slide 21

This figure is from a reference, the *Culture of Animal Cells*, by Ian Freshney. Many labs have multiple laminar flow hoods and other equipment near incubators, which generate vibration. This can lead to patterning in culture vessels, such as in this slide. The use of vibration-dampening tables can avoid this contributor to variability. Though not shown on this slide, vibration can also be a problem with individual cell culture inserts.

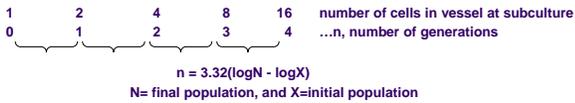


Cell Age

- Cell Characteristics can vary over time
- Use Standardized Protocols to control and understand the age of your cells



Population Doublings, Generation Number:



Passage Number:

- Number of times a culture has been subcultured or split
- Does not reflect population age unless there is a *consistent time* between splits and a *unvarying split ratio*



Kruse and Patterson, Tissue Culture Methods and Applications, 1973

Slide 22

Cells can vary over time in their characteristics, such as expression patterns and karyotype. There is a normal karyotype here on the right-hand side of this slide, and it's important to understand and control the age of your cultures. Generation time or population doublings, gives an accurate measurement of the age of your cells. I have included the formula for calculation. I would take a look at this after, and start using it in your lab. It does require the counting of the number of cells at the cell seed, and also at the cell harvest. Many labs report passage number. Every time a vessel is subcultured, the passage number is increased by one. In order for this method to give a reliable and reproducible measurement of cell age, the time between splits must be consistent, and the split ratio – for example, one flask split into three flasks – must be unvarying. Any alteration will impact the cell age. Everyone in your lab must use the same protocol in order for this to be used as a predictor for cell age. This is a much less accurate method of measurement.

Substrates

Vertebrate cells have negative surface charges, and can be cultured on either positive or negative charged surfaces

- Glass and Tissue Culture-Treated polystyrene plastic carry a negative charge
- BD Primaria™ carries both positive and negative charges
- Polylysine surfaces have positive charges

Extracellular Matrix components (2D or 3D)

- Cells spread using the ECM they lay down and the serum or media derived attachment factors
- Fibronectin is key attachment factor derived from serum
- BD BioCoat™

Microporous membranes (cell culture inserts)

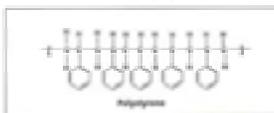


Slide 23

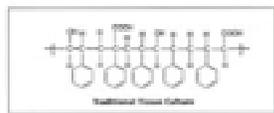
BD provides many products that can be used to control the substrate that your cell sees. Vertebrate cells have negative surface charges and can be cultured on either positive or negative charge surfaces, depending on the cell morphology and phenotype that you are looking for. Glass and tissue cultured treated polystyrene plastic carry a negative charge. Other alternatives such as Primaria and Polylysine can change that charge, and allow for different effects on your cells. There are also extracellular matrix components that can be used to alter the way in which the cell interacts with a plastic surface. Some are in two-dimension – some provide three-dimensional surfaces. Microporous membranes, cell culture inserts, are also used as a device to allow apical and basal-lateral contact with a liquid environment.

Substrates: Polystyrene

Untreated polystyrene



Vacuum-gas plasma treatment- oxygen



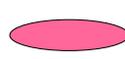
Native polystyrene is rendered **hydrophilic** by a process that adds a variety of **negatively charged functional groups** to the surface.

BD Falcon™ Cultureware is **vacuum-gas plasma treatment** under specific conditions of pressure and temperature...

Non TC PS



TC PS



Slide 24

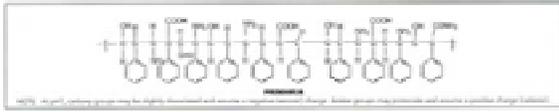
Polystyrene is used in cell culture due to its crystalline characteristics that make it optically clear. Cell culturists like to be able to see their cells. However, negative – native polystyrene is positively charged. The cells are weakly adherent to the untreated polystyrene surface. On the left-hand side is a graph demonstrating what the cells will look like on a non-TC treated polystyrene. Manufacturers of cell cultureware treat the surface of vessels with what is referred to as tissue culture treatment. This adds negative charged – negatively charged functional groups to the surface of the plastic. At BD, we use vacuum gas plasma treatment under controlled pressure and temperature, to change the surface to a more negatively charged surface, allowing cells to attach and spread on the surface. On the right, a more spread schematic on the slide demonstrates this modification in cell attachment.



Substrates: Polystyrene

Other Modifications to Polystyrene

Vacuum-gas plasma treatment- oxygen + ammonia



BD Primaria™ is a permanent, stable cell culture substrate with a more complex surface chemistry, integrating various amine and amide functional groups into the traditional hydroxyl/carboxyl tissue culture surface chemistry by using a gas mixture containing oxygen and ammonia.

Some BD BioCoat™
Extracellular proteins
BD Primaria™



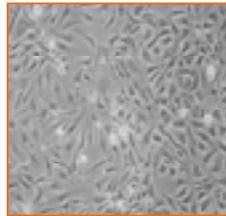
Slide 25

There are also other modifications that can be made to the polystyrene surface, such as the addition of extracellular matrix proteins, Polylysine, a synthetic polypeptide, and Primaria, a modification of plasma deposition using alternative gases. These surfaces allow a closer adhesion of the cells to the surface, which is illustrated by the graphic at the bottom of the slide.

Human Umbilical Vein Endothelial Cells (HUVEC)



TC Plastic



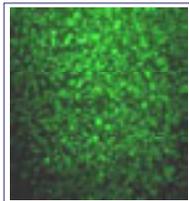
BD BioCoat™ Collagen I



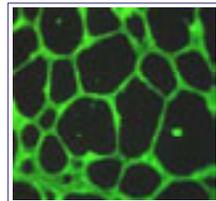
Slide 26

Here is an example using human umbilical vein endothelial cells. On the left are cells seeded onto TC treated polystyrene. On the right, BD BioCoat™ Collagen I. Same cells, same media, different matrix. The cells attach better to collagen. But be aware when you change the matrix, you may also be changing the selection population, or the phenotype of the cells, such as the digoxin Caco-2 cell example I showed you earlier.

Human Microvascular Endothelial Cells



Collagen I
2D



BD Matrigel™
3D

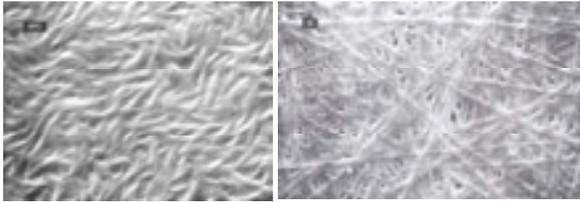


Slide 27

Another example of substrate effect are these human microvascular endothelial cells. On the left, the cells are plated onto Collagen I. The cells attach, spread, divide, and express a proliferative phenotype. On the right, BD Matrigel™, a three-dimensional matrix protein. Here you can see the formation of endothelial tubes. This is commonly used by pharmaceutical companies to screen for angiogenic compounds. Same media, same cells, different matrix.



BD BioCoat™ Collagen



Amorphous

Fibrillar



Slide 28

This slide is here to show you an example of a two-dimensional matrix on the left, and a three-dimensional matrix of the same protein on the right. Both are BD BioCoat™ Collagen I. On the left is amorphous Collagen I, providing a flat, even two-dimensional surface. On the right is fibrillar collagen, manufactured using a method that allows the formation of three-dimensional collagen fibers, similar to those seen *in vivo*. Two different surfaces prepared using the same Collagen I protein, allowing the cells to express two different phenotypes.

Adherence of HEK-293 Cells to BD BioCoat™ Assay Plates



HEK-293 cells were cultured under serum-free conditions on TC-treated or BD BioCoat™ PDL 384-well black/clear plates. Samples were washed using a Skatron Washer (Molecular Devices)

Slide 29

This is an example of the use of a substrate to control variables in experimental data. When HEK-293 cells are cultured on TC treated plates in serum free media, they are weakly adherent. This becomes apparent after a wash step – a fairly common procedure in cell-based assays. We have used the fluorescent dye calcein, staining the cells to allow quantification of the cell loss by fluorescence. Although the cells are attached before the wash, you can see that the wash step has rinsed away the cells. When these same cells in serum free media are plated onto BD BioCoat™ Poly-d-lysine as a modification of the polystyrene, the cells adhere and stay attached following a rigorous wash. These types of HTS assays that require rigorous washing – it becomes important to understand what the cells will do in response to your procedure.

Morphology: Phenotype

Primary Rat Hepatocytes



Col I (2D thin coat)

Col I (3D gel)

BD Matrigel™



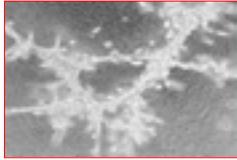
Slide 30

I would like to talk a little bit about cell morphology. I have a couple of examples of how morphology and phenotype are tied together. On our website, bdbiosciences.com, you can find technical bulletin no. 420, where the P450 functional data relating to these photographs is reported. On the left side of the slide are primary rat hepatocytes, plated onto a thin coat Collagen I, similar to the earlier slide I showed you with amorphous and fibrillar collagen. The hepatocytes are spread and attached, and express a proliferative phenotype. On the right are these same cells, same media, plated onto BD Matrigel™. The matrix provides a 3D environment to the cells, which are rounded and form clusters suggestive of ducks that are expressing a quiescent, differentiated phenotype.



Morphology: Phenotype

Aortic Smooth Muscle Cells



GFR BD Matrigel™



Collagen I



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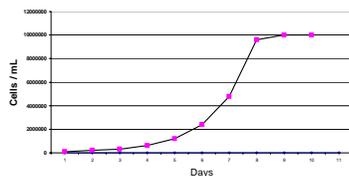
This second example is of aortic smooth muscle cells, and is from technical bulletin 425. On the left are cells plated on growth factor reduced BD Matrigel™, providing a 3D surface. These smooth muscle cells are not dividing, and express a contractile phenotype. On the right are the same cells plated onto thin coat Collagen I. The cells are expressing a proliferative phenotype. Technical bulletin 425 reports some immunohisto chemistry data that support this conclusion.

Routine Maintenance

- Medium changes
- Monitor cell morphology
- Stay on a strict schedule
- Subculture at 80% confluence



Representative Cell Growth Curve



Slide 32

Routine maintenance will help to standardize data. Medium changes and subculturing should be done on a regulated schedule, and should be done in a reproducible manner. The graph at the bottom of this slide is representative of a semilog cell growth curve, which demonstrates lag, exponential growth, and contact inhibition of cells at saturation density. Many cells will phenotypically shift if allowed to reach saturation density. Cells should always be subcultured when they are in the log phase of growth.



Dissociation Techniques

Mechanical

- Cell scraper, trituration
- Colony division, scalpel

Enzymatic

- Trypsin, Collagenase, Dispase, Pronase
- Rinse with Balanced Salt Solution without Ca⁺⁺ Mg⁺⁺
- Enzymes should be aliquoted and frozen at 20°C

Chelating Agents

- Citrate and Ethylene-diamine-tetra-acetic acid, (EDTA, Versene)

Calcium and Magnesium are important regulators of cell to cell junctional complexes



**Standardized Routine Techniques
When and How**



Slide 33

Dissociation methods can also affect the quality of your data. I will go over three methods that are routinely used. First is mechanical dissociation. This is done using a cell scraper, shown on the right. This mechanical method is used for passage of embryonic stem cells, generally using a scalpel to divide the colonies. The advantage to this technique is that the cells are not exposed to enzymes that can sometimes strip receptors from the cell surface, and can induce changes in phenotype. The disadvantage can be that cell scraping and trituration can sometimes result in debris, and it is difficult to result in a single cell suspension. Enzymatic dissociation is generally done using Trypsin, Collagenase, Dispase, or Pronase. This allows the cells to break from each other, and from the substrate. The cells are usually rinsed prior to treatment using balanced salt solutions without calcium and magnesium. Calcium and magnesium are important regulators of cell-to-cell junctional complexes. At the bottom is a nice electro-micrograph of Caco-2 cells with junctional complexes. Rinsing without calcium and magnesium allows the cells to begin to break from each other. This lessens the time and concentration that the culture needs to be exposed to the harsh enzymatic treatment. Enzymatic dissociation can result in damage to cell membranes, and result in poor attachment, clumping, and ragged membranes. Enzymes should be aliquoted and frozen at -20°C, to maintain maximal effectiveness. Finally, chelating agents such as Citrate and Ethylene-diamine-tetra-acetic acid, (EDTA, Versene) are used in the same techniques. Remember – standardized, routine techniques.

Cryopreservation

Cell Response to Cryopreservation:

- Reduced Metabolic Activity
- Ionic balance and Osmotic balance are disrupted
- Free radicals accumulate
- Ice forms around the cell
- Molecular stress responses are initiated

Solutions:

- Tissue Culture Media
- Serum (10-90%)
- Proteins such as albumin
- Cryo-protective agents
 - Dimethylsulphoxide (DMSO)
 - Glycerol
- Vitrification



Baust, JG, Baust, JM, Advances in Biopreservation, 2007

Slide 34

I'd like to touch briefly on cryopreservations. Many cells respond to cryopreservation by reduced metabolic activity, ionic balance and osmotic balance are disrupted, free radicals can accumulate, ice forms around the cells, and molecular stress mechanisms are initiated. Currently, most labs are using solutions to this problem that include tissue culture media, supplemented with serum at 10% to 90%, and proteins such as albumin. Many labs are using cryo-protective agents such as DMSO and Glycerol. Some labs are beginning to use vitrification. It is important to understand that the techniques that you are using to cryopreserve your cells may be affecting the resulting cell phenotype, and is another variable in standardizing your cell culture techniques.



Contamination

Contamination Sources:

- Chemical, endotoxins, plasticizers, disinfectants, fluorescent lighting
- Biological: bacterial, viral, fungal, mycoplasmal, cellular



Slide 35

I'd like to end the seminar by discussing contamination. There are two sources of contamination – chemical and biological. The ATCC reports that 11% to 15% of cell lines in the U.S. are contaminated with either biological or chemical contaminants.

Contamination: Chemical

- **Endotoxins:** lipopolysaccharide containing by-product of gram-negative bacteria
 - Water, sera, culture additives
- **Plasticizers:** in storage containers, tubing
- **Reused storage containers**
- **Disinfectants:** deposits left from washing glassware
- **Germicides:** used to disinfect Incubators
- **CO₂:** Use medical grade



Slide 36

Endotoxins are the byproduct of gram-negative bacteria that is present in water, sera, and culture additives. Endotoxins are frequently introduced from poorly maintained water systems, such as ion exchange resins. Plasticizers are used in storage containers and tubing. Use medical grade of plastics or plastics that are intended to be used in cell culture lab – laboratories. Reused storage containers can influence the way the contaminants leach from plastic into your media. Disinfectants commonly leave deposits on washed glassware, and germicides are frequently used to disinfect incubators. Always use medical grade CO₂. Something to remember is that serum proteins can bind chemical contaminants such as heavy metals, so switching to serum free media can unmask a toxic chemical contamination.

Contamination: Biological

- **Bacteria, Mold, Yeast**
 - Easily detected, pH, turbidity, cell morphology better to avoid antibiotics
- **Viral, Mycoplasma**
 - Alter host cell function, hard to detect
- **Cell Cross-contamination**
 - Technique, don't borrow cells

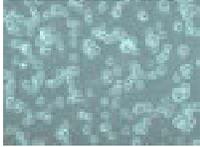


Slide 37

There are three major groups of biological contamination – bacteria, mold, yeast, which are all easily detected. There are viral and mycoplasma contaminants, and there is also cross-contamination with other cells. Cross-contamination is easily avoided by not borrowing cells from your neighbor, and using good technique. I'll also again direct you to the ASCB July newsletter, where there is a brief but comprehensive overview of control of cross-contamination.

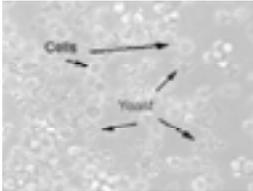


Contamination: Biological



Yeast

- Does not cause early pH changes
- Medium becomes cloudy
- Later, yeast can cause media to become basic (purple media)
- Distinctive bread-like odor (though it isn't a good idea to sniff your cultures)



www.biochemweb.org

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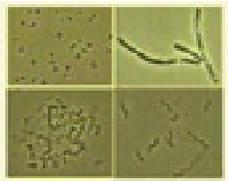
Here's an example of yeast, which does not cause early pH changes. The medium becomes cloudy, and later, yeast can cause the media to become basic. Distinctive bread-like odors are apparent, although it's always not a good idea to smell your cell cultures.

Contamination: Biological

Bacterial

Causes changes in pH of Medium

- Medium becomes acidic
- Cloudy and bright yellow
- Often produce toxins that destroy cells



The Microbial World, Jim Deacon
Institute of Cell and Molecular Biology, The University
of Edinburgh



Slide 39

Bacterial contamination does cause changes in pH of the media. If you have an indicator of pH, your media becomes acidic, and becomes cloudy and bright yellow. On the right is an example of media in which we induced a contamination event. This example has both bacterial contaminant in the media, and a mold at the liquid/air interface. These often produce toxins that destroy cells.

Contamination: Biological

Mold

Does not cause immediate pH changes

- Often is not cytotoxic
- Easy to observe under a low power microscope and can even be seen without magnification in advanced stages of contamination
- Tough to catch early
- Appears whiteish, yellowish, or black in culture



Slide 40

Mold does not cause immediate pH changes, is often not cytotoxic, but is very easy to observe under low-power microscopes, and can even be seen sometimes without magnification in advanced stages of contamination. It's tough to catch it early, but easy to catch it late.



Contamination: Biological

Mycoplasma

Cannot be seen under normal magnification

- No overt effects in culture, only subtle ones
- The only way to confirm mycoplasma contamination is by routine testing

Mycoplasma can cause changes in:

- Cell growth characteristics
- Cell metabolism
- Disruption of nucleic acid synthesis
- Chromosomal aberrations
- Changes in cell membrane antigenicity
- Can alter transfection rates and virus susceptibility



Slide 41

Mycoplasma, on the other hand, cannot be seen under normal magnification. It has no overt effects in culture – only subtle ones. The only way to confirm mycoplasma contamination is by routine testing. It is caused by technical handling, serum, and other sources that are introduced in your media. Be sure to buy serum from a manufacturer who certifies it as mycoplasma-free. Mycoplasma can cause changes in cell growth characteristics, cell metabolism. It can disrupt nucleic acid synthesis. It causes chromosome aberrations, and changes in cell membranes. It can also alter transfection rates and viral susceptibility. There are many kits available to bring testing procedures into your lab, and also testing services that will provide you with the latest and most comprehensive testing available.

Contamination: Control

Control

- Certified seed culture
- Aseptic technique
- Periodic testing
- Antibiotic-free media
- Sterile equipment, glassware and media
- Quality reagents
- Use frozen cell stock

Most Dangerous:

- Undetected contaminants



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So to wrap up, how do you control for contamination? You use a certified seed stock, a septic technique, periodic testing, antibiotic free media, sterile equipment, glassware and media, and quality reagents. We use a frozen seed stock in our lab. Whenever a new cell type comes into our lab, we set up a seed stock that generally – and go back to that seed stock periodically, so that our cells are never out of the lab – out of the liquid nitrogen for more than four to six weeks at a time. Most dangerous to you is undetected contaminants, which cause incorrect interpretation of your results.

Aseptic Technique



- Gown appropriately
- Frequently disinfect gloved hands with 70% ethanol
- Disinfect your work surface before you begin
- Laminar Flow hoods provide a physical barrier to contamination
 - HEPA filters trap airborne contaminants
 - Blowers move filtered air at specified velocities in a non-mixing (laminar) stream across a work surface
 - Not 100% sterile and are not replacement for good microbiological aseptic technique
 - Do not overcrowd work space
 - View window should be lowered to the proper operating height of 8 inches
 - Before use - allow blower to run 10 min. to filter the cabinet air of any particulates
 - Recertify / Replace HEPA filters at 1000 hours
 - Periodically disinfect the catch basin
 - Test with T-Soy and Sab-Dex settling plates
 - Use UV light before and after work



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I've included this slide here just so that you can see some quick tips on aseptic technique. You always want to make sure that you are gowned appropriately, that you disinfect your gloves with 70% alcohol, that you disinfect your work surface before you begin, and make sure that you understand the limitations of your laminar flow hood. It does provide a physical barrier to contamination, but it needs to be adequately maintained in order to make it effective. So you want to make sure that you always use clean, cuffed laboratory coats and latex gloves, tie long hair back, and avoid moving material or hands in and out of the cabinet as much as possible.

Experimental Data

Experimental data is influenced by:

- Cell Source
- Initial Growth Conditions
- Medium Composition
- Cell Counting Procedure
- Physiological Parameters
- Characterization
 - Age, Karyotype
- Substrate, Matrix, or Not
 - Morphology
- Dissociation Method
- Contamination Control
 - Aseptic Technique
- Cryopreservation



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Experimental data is influenced by many things – cell source, your initial growth conditions, your media composition, physiological parameters such as pH and temperature. You can always characterize your cell type by age and karyotype. You can modify your morphology and phenotype by your substrate, and whether you use a matrix or not. Be sure to control your dissociation methods and your contamination control. Use aseptic technique, and be aware of the effects of cryopreservation. Any of these can influence the variability of your data. Standardization is really just good laboratory technique.

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So on this slide here, we've given you the contact information for the BD Technical Support group, and also my contact information. Feel free to give us a call if we can help you at any time. Thank you very much.

