OVIZIO IMAGING SYSTEMS

ASSESSING THE HEALTH STATUS OF YOUR CELLS

Accuracy and Precision in Suspension Cell Viability Monitoring

Abstract

At Ovizio, we are frequently asked why we chose double digital differential holography microscopy (D3HM) to monitor cell viability. We thought that the most comprehensive way to approach an analytical procedure was to question every aspect of it as a validation method. We asked ourselves: What do we want to measure? How can we measure it? How can this be achieved accurately and precisely? What are the techniques involved? Which one is the best? By reading the results of our study, you will get:

- A clear understanding of the meaning of accuracy and precision in cell viability
- An overview of the principles of the different methods, their advantages and limits
- An insight comparison of the techniques and instrument monitoring in cell viability in terms of their accuracy and precision

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Cell Viability

Together with cell concentration, viability is one of the key parameters of a cell culture process. Any optimization in the cell growth kinetics or in the process operation timing can ultimately lead to a dramatic increase in the final production yield.

During process development, viability data allows bio-engineers to clearly define the best parameters (such as dissolved oxygen, pH and nutrients) related to cell growth and to establish critical process parameters such as determining feed or harvest times. These parameters are the core of the QbD (Quality by Design) approach.

In cGMP operations, monitoring cell viability provides key information on the status of the culture. This helps calculate specific production and consumption rates, quantify volumetric productivity of a batch and indicate a culture's infection. It is also a critical element according to EMEA or FDA regulations for documenting and continuously controlling the cultivation process, which is the key principle of Process Analytical Technology[1].

Cell viability monitoring implies the design of an optimal analytical method that will enable the most accurate and precise measurement (Figure I).

If accuracy represents the closeness to the target, precision is a matter of reproducibility. It requires the measurement of a relevant parameter, capturing and interpreting the related signal as well as being statistically representative.

Therefore, it is crucial to clearly understand what we want to measure and how the available techniques can best evaluate the accuracy and precision of the method.

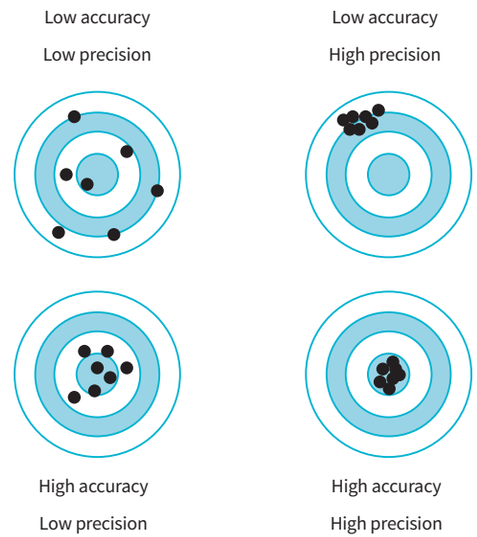


Figure I: Accuracy vs precision

Cell viability is defined as the ratio between living cells and the total number of cells (dead/dying and living). The main challenge lies in correctly counting a group of cells and distinguishing between live and dead cells. It has been shown that different Programmed Cell Deaths (PCD) have a Point of No Return[2,3,4], meaning that prior to this point a "dying" cell can go back to a normal activity.

Cell death and the apoptotic pathway is induced by two main mechanisms, receiving either an intrinsic or extrinsic signal, (e.g., stress factors or ligand binding on the membrane); however, a cell is capable of halting cell death due to cell signal regulation and/or removal of apoptotic inducers[5].

We provide a quick overview of cell death here, but it should be noted that there are many causes and pathways that exist, and the true "point of no return" is still debatable. Cell death can occur after the introduction of stress, senescence of the cell, an infection or a death signal via membrane-ligand binding. A living cell will either proceed to one of the many death pathways, be ingested or be mechanically destroyed. In the first case, this will lead to a degradation of its organelles, the insertion of pores into its cytoplasmic membrane and finally the destabilization of both nuclear and cytoplasmic membranes (Figure 2).

With regards to cell viability measurement, the method is accurate if it has the ability to identify a cell as "dead", which is the moment when it can no longer return to a living state (passes the point of no return). It is precise if it gives reproducible results and is always able to identify dead cells from a certain step in its death pathway.

The purpose of the following segments is to explore and understand the principle of the different methods available and accordingly review the accuracy and precision of current instruments applying said methods.

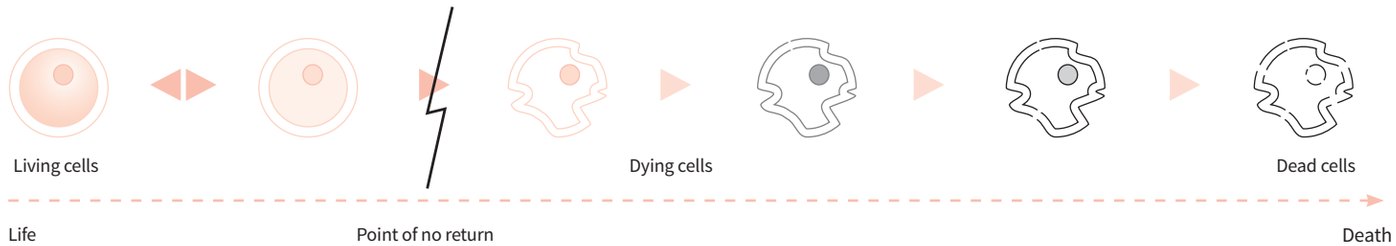


Figure II: Schematic representation of Cell Death

State of analytical methods

There are several analytical methods for the measurement of cell viability. Their differences essentially arise from the nature of their observation and the technological choice. Depending on the selected technique, it will be more or less easy to perform the analysis, interpret the results and process the data. As most of the technical principles have been known since the first decades of the 20th century, recent technological evolution, such as miniaturization and automation as well as new computing methods, has increased the accuracy and precision of cell viability measurement methods.

To review each method individually, it is essential to first explain the available options and their implications by going through 4 steps: which cell death characteristics are measured; how are they measured; what is the available information and how to process it; and finally, how to sample or visualize the culture.

How to characterize dead cells

There have been numerous scientific articles and publications characterizing and identifying key attributes (morphologically and otherwise) of apoptosis and its inducers. From these studies, various parameters that characterize the cell status (living/dying/dead) have been concluded. In this section, we will quickly describe the three principles upon which the main methods are based: membrane integrity, cell profiles, and labelling.

Membrane integrity

A dead cell will not maintain its membrane integrity due to the formation of pores around the membrane and by disintegration of its unity (Figure II). Techniques focused on this property lack accuracy, as many pathways lead to cell death and some dying cells might overstep the Point of No Return before the formation of pores. However, the precision of such techniques can be very high as there is little ambiguity about the presence/absence of pores and non-uniformity.

Cell profiles

Morphological changes of a cell throughout its life cause it to progressively fall apart; therefore, a cell may be characterized by its shape and granularity. The accuracy depends on the profile setting of a dead cell and consequently is impacted by a pre-established choice.

Labelling

A completely different strategy uses direct labelling, such as a fluorescent or radioactive molecule coupled to an antibody that specifically binds to an antigen of interest, or indirect such as genetically manipulated cell labelling. These techniques are frequently used in vitro. Their principle induces a bias as it involves either modifying the cell's DNA or labelling it with a targeting agent. As the cells are not in their natural state, the accuracy of the techniques that rely on this tactic is poor. Precision will widely depend on the type of labelling used.

Labelling will not be discussed any further in this paper.

How to acquire the related data

Now that the main parameters that characterize a dead cell have been described, the next step is to discuss how they are measured. These methods have evolved into different paths over the years: from the old-school yet pertinent capture of an image of the culture (or a sample), to the application of the binary Coulter principle, or the use of the comprehensive double differential digital holographic microscopy. They can be classified according to the parameters they measure.

Techniques for measuring membrane integrity

Trypan blue

First synthetized in 1904 by Paul Erlich (Nobel Prize in Physiology or Medicine, 1908), Trypan Blue is one of the most-used dyes in laboratories for cell viability monitoring. Its name is derived from its ability to kill trypanosomes (responsible for sleeping sickness).

Trypan Blue is capable of entering (almost exclusively[6]) a dying or dead cell through pores generated within the outer membrane following programmed cell death and therefore, is incapable of entering living cells. The appearance of plasma membrane pores is therefore the moment when a cell is identified as "dead", whenever Trypan Blue is used and regardless of the process. It is interesting to note that this event occurs after the point of no return is reached, therefore some dying cells are not revealed by the technique but every revealed cell is either dead or dying with no possible return. The techniques involved are precise but lack accuracy. Moreover, the sample is wasted.

Coulter principle

The Coulter principle is based on the fact that a living cell, being an electric micro insulator, creates an impedance when submitted to a low electrical current. This principle is used primarily for cell counting and size measurements and not for viability measurements specifically. However the Electrical Current Exclusion principle[8] brings another dimension to cell counting. Dead cells let the current pass due to destabilization of their membrane integrity, whereas living cells do not. The techniques involved are precise but lack accuracy as they quantify only the dead cells well advanced in the death pathway.

Wallace H. Coulter described his principle in the 40s in the purpose of quicken blood cells analysis. Since then, it has been used in many applications in the medical world and in research.

Techniques for measuring cell profiles

Microscopy

Image capture may also be done without staining. Microscopy is used here only to visualize the shapes of the cells in the best possible resolution. The culture (or a sample) is subject to a light beam in order to be photographed and/or filmed. When the whole culture is illuminated, a red LED light is often used to avoid disturbing the cells. The techniques involved are less precise but can show greater accuracy depending on what death pathway the cells use.

Flow Cytometry (FACS)

Fluorescence-activated cell sorting (FACS) provides a method for sorting a heterogeneous mixture of cells into two or more containers, one cell at a time. The working principle behind this method is based on specific light scattering and fluorescence patterns of each cell.

The technique was expanded by Len Herzenberg, who was responsible for coining the term FACS[9], but FACS is trademarked and owned by Becton, Dickinson and Company.[10]

Specifically, in suspension cell culture, cells are entrained in the centre of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. The flow passes through a fluorescence measuring system where different patterns or characteristics can be detected, identified and separated. Because there are numerous applications for FACS, it is still a common method for cell culture monitoring and counting.

Techniques for measuring the cell's profile and membrane integrity.

Holographic Microscopy

Dennis Gabor (Nobel Prize in physics in 1971) discovered the principle of holography in 1947. The first holograms were only created in 1964, with the development of laser technology. Today, holography can be achieved with different light sources such as LED light.

The deformation of the membrane due to inserted pores changes the way a cell interacts with light. The shape and size are different but other details are also impacted and can be studied with adequate equipment such as the phase shift of the wavelength of light going through a transparent object. Whereas classic Light Microscopy (LM) can only see light wavelength, Double Differential Digital Holographic Microscopy (D3HM) can also see Optical Path Length (OPL, a value depending on the refraction index of the objects the light crossed) by comparison between the light beam and a reference light beam. The techniques involved focus on membrane pores insertion and cell profiles, combining their accuracy and precision.

How to analyze the acquired data

Once information about the culture is acquired, data needs to be analyzed. While it is essential to have a sensitive technique with good accuracy and precision, it becomes pointless if the acquired data cannot be efficiently processed. Since different methods detect death at different stages of the cell death pathway, they have different thresholds and can indicate different ratios at a given time.

Manual Analysis

The most basic and quick way to count cells and to calculate viability is performed manually. However, samples need to be diluted, and these dilutions can lead to inaccurate measurements and calculations. Further description of manual sampling effects is described later.

Once the (colored) sample is correctly displayed between the microscope slide and the coverslip, the number of uncolored cells is divided by the total number of cells (colored and uncolored) and then multiplied by one hundred to obtain the percentage of viability.

The limitation of this technique is inherently linked to its manual aspect: it is not feasible for a human to count thousands of cells. So, the sample and number of events has to be small, thus introducing a bias from the handling and required dilution (described above). This leads to low precision, subjectivity, operator-dependent results and poor accuracy. The estimated error for manual cell counting is summarized by the following equation[11]:

$$Error\ max = 2x\ \frac{100}{\sqrt{n}}\ \%$$

n= Number of counted cells

This gives an error of 20% for a count of a hundred cells. The manual analysis is of course the weaker way to process data. Indeed, the most precise technology seems pointless if compromised by the various interpretations of different users. The large percentage of variation between two different people cannot be ignored when comparing methods of monitoring cell viability. The lack of reproducibility and objectivity leads to lower precision.

Image Recognition Software

Image Recognition Software (IRS) automatically detects patterns in datasets and uses them to characterize new data[12,13]. It allows the distinction between dead and living cells by their shape, (artefactual) color and granularity.

IRS works by identifying regions of interest (ROIs) or "objects" present in the picture or movie. Measurements and statistics can be collected from the object (the cell's image), such as (artefactual) color, intensity, shape, size, position and potential other parameters as well as the number of objects and their distribution. According to the used technique and the obtained values, the cell is then considered either alive or dead.

IRS-based analyses have the major advantage of enabling machine learning in order to improve the subtlety of their discrimination. This leads to high precision. The accuracy, on the other hand, is impacted by the predefined "image" of dead cells implemented into the software. This subjective choice lowers the accuracy of the techniques involved except for the ones based on membrane integrity. These have their accuracy objective (presence/absence of pores in the membrane) as it is dependent on the accuracy of their primary assumption.

Specific software analyzing raw data

Many of the previously discussed techniques have very specific analysis approaches. For example, a software distinguishing between the impedance of living and dead cells can process variations of the recorded impedance, transforming electric current into digital data. Specific software is also used by flow cytometer and Double Digital Differential Holographic Microscopes to transform the raw data they capture into relevant information on the culture.

Specific software analyzing raw data are all based on membrane integrity and are fully automated. With no intervention of human decision, strong accuracy and precision are ensured, except in the case of Flow Cytometry, where setting and gating are manual, leading to a lack of both accuracy and precision.

Moreover, the OPL-analyzing algorithms combine full automation of specific software analyzing raw data and machine learning. This brings a high score in accuracy and precision.

Machine learning

To improve efficacy, Image Recognition Software and OPL-analyzing algorithms use Machine Learning. It is a self-improving system capable of optimizing software that distinguishes between objects thanks to pre-entered parameters and a calibration phase where the system analyzes known objects to "learn" how to automatically recognize them[12]. It can be considered as the automation of the optimization. As a result, analysis becomes more and more fine-tuned over time. This can be implemented on only one machine but some companies take the bet of offering participative machine learning to enhance their solution by combining the results of several machines.

How to sample

Now that each step of the data acquisition and analysis has been described, another important matter needs to be discussed: does the data represent the whole culture or only a sample? The way suspension cell culture is sampled also depends on the chosen technique and the kind of culture. The three different approaches are: manual, on-line and in-line sampling. The aim of automated sampling (the latter two) is to avoid bias in measurement and contamination. But in the context of cell culture, where some processes are applied to small volume, in-line techniques have the advantage of being free of sampling.

Sampling also encompasses sample preparation as for instance, brings the cell density to the measurement range of the instrument used. Every alteration of the sample comes with a loss in quality of the sample, thus making it more and more different from the original volume present in in the original cell culture vessel.

Manual Sampling

The first sampling method ever used was manual. While it is still mostly used, it represents an important source of bias and a significant potential source of contamination of the culture (the sample may not be homogenous or representative and the invasion of the culture media may disturb the cells). As it is user-dependent and sometimes not made in a homogenous way, it lacks accuracy and/or precision.

On-line techniques

On-line techniques are not based on the same principle, acquisition methods nor analysis but have common advantages as they:

- are directly connected to the cell culture vessel
- bring limited risk of contamination
- eliminate the human cause of errors

For some cell imaging strategies, the sample may be cyclically extracted from the bioreactor to be conveyed to the microscope.

On-line techniques waste the sample used, which can be impairing in cases where the product is a small volume, but they are free of contamination risks.

In-line techniques

All In-line techniques are not based on the same principle, acquisition methods nor analysis but share the particularities of:

- being directly connected to or integrated to the culture vessel
- very limited or no risk of contamination depending on the method
- using automated sampling or having no need to sample at all
- in the earlier case, “recycling” the sample so there is no material waste.

In-line techniques can be even more accurate and precise than on-line techniques as they have a more representative view of the culture.

Overview of today's monitoring techniques

Now that we understand the main principles currently used in the cell culture world, we can review and classify the main available techniques for cell viability monitoring by considering that the most accurate technique must be based on cell profiles and the most precise must be focused on membrane integrity.

Both accuracy and precision are higher with automated analysis tools coupled with machine learning. Lastly, in-line techniques are the best option for accurate and precise sampling and ensure neither waste nor disturbance of the culture.

Techniques using Trypan Blue

Hemocytometer

Hemocytometer sets its sight on the cell's membrane integrity to discriminate dead from living cells. It uses the Trypan Blue property to color dead and dying cells. It is then displayed between a microscope slide and a coverslip. The microscope slide is graduated, allowing the user to have a precise idea of the volume of the sample observed. The viability is calculated via manual counting and visual discrimination. The sample visualized with the hemocytometer is manually extracted from the culture.

The hemocytometer is certainly the most-used and the easiest solution to implement in labs. However, it is time consuming in terms of workload and demonstrates high variability between measurements as it lacks precision and accuracy due to the manual acquisition, analysis and sampling, quantifying only the dead cells well advanced in the death pathway.

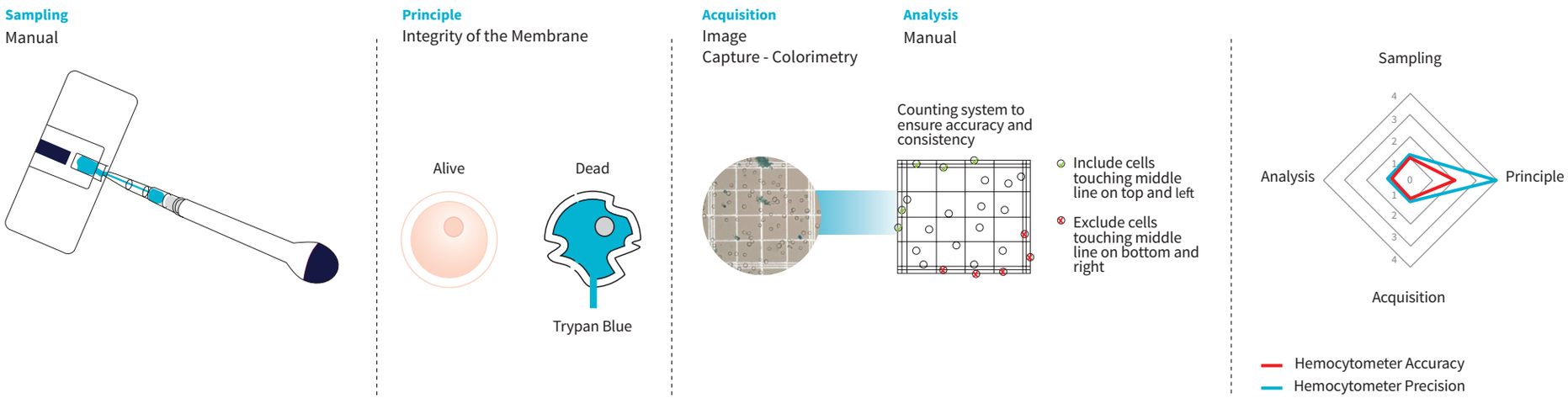


Figure III: Hemocytometer

Image mosaic

The image mosaic technique focuses on the cell's membrane integrity to tell dead from living cells. Unlike the hemocytometer, several slides are used and are imaged. The images are then sent to a computer to be analyzed by a specific software. Image mosaic software aims to overlap those pictures to build an image mosaic of all cells on the microscope slide grids.[7] It is then processed by an IRS. The samples visualized with the image mosaic technique are manually extracted.

Image mosaic is an addition to the hemocytometer as it increases its precision thanks to automated acquisition and analysis but it still suffers in precision and accuracy due to manual sampling. It also only quantifies the cells well advanced in the death pathway.

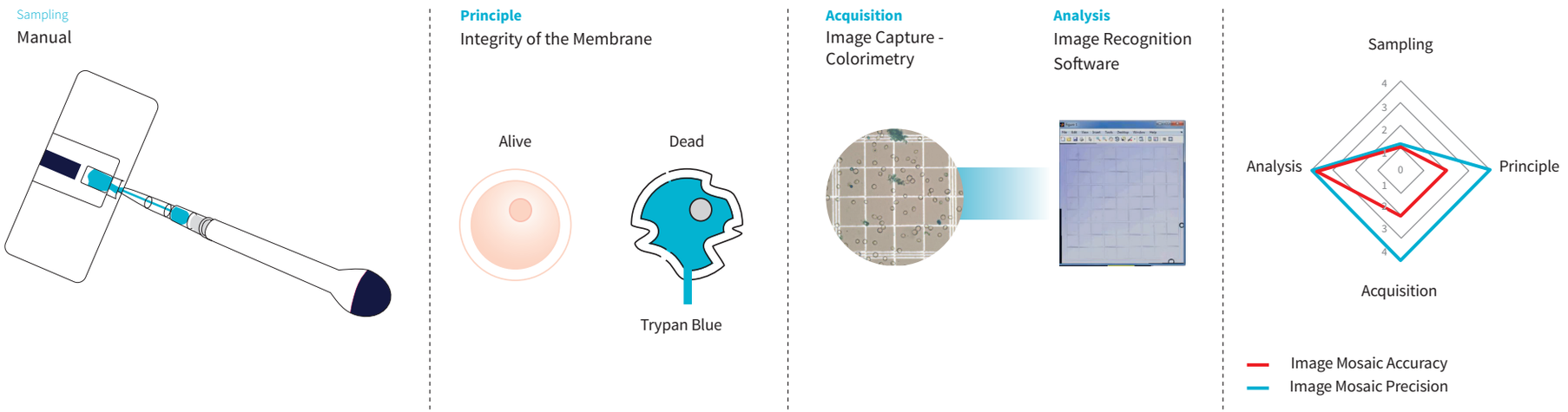


Figure IV: image Mosaic

Cell Imaging System

Membrane integrity is also used for the characterization of dead cells. Here, the coloration of the sample by the Trypan Blue is performed automatically. Once cells are mixed with the dye, they are drawn into the fluidics system of the machine and imaged. The cell imaging software uses the photographs taken by the microscope and analyzes them by an IRS. CIS may get the sample manually or cyclically extract it from the bioreactor to be conveyed to the microscope (on-line sampling).

The cell imaging system is the almost fully-automated version of the hemocytometer. In most cases, it still lacks precision and accuracy because of its manual sampling. However, some devices are now equipped with an automated sampling version that allows online monitoring. As it depends on the use of Trypan Blue, cells cannot be returned to the culture, thus the sample is wasted.

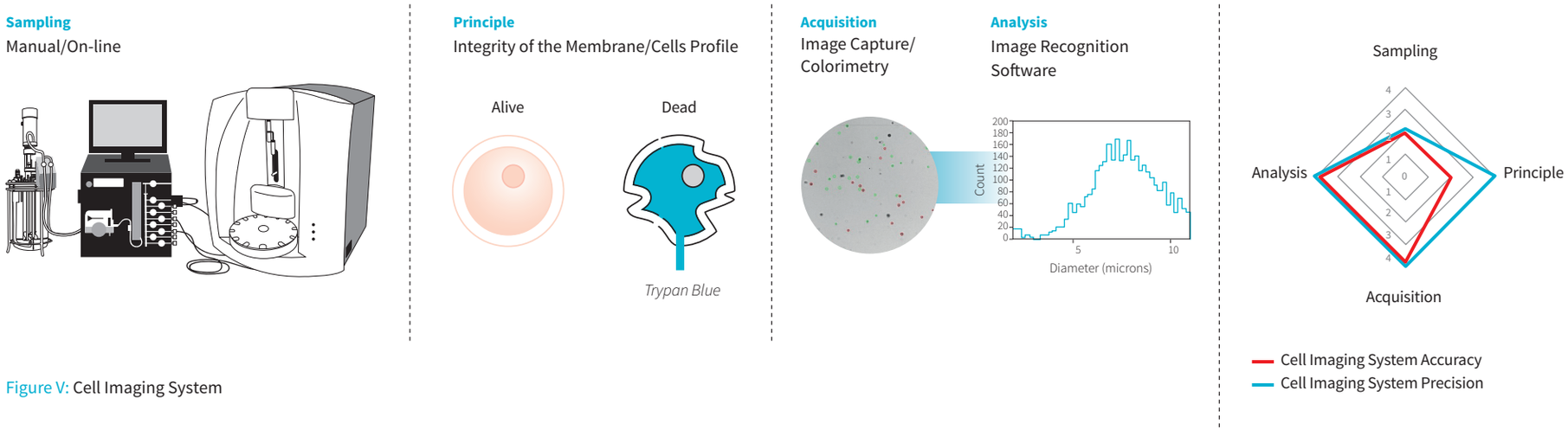


Figure V: Cell Imaging System

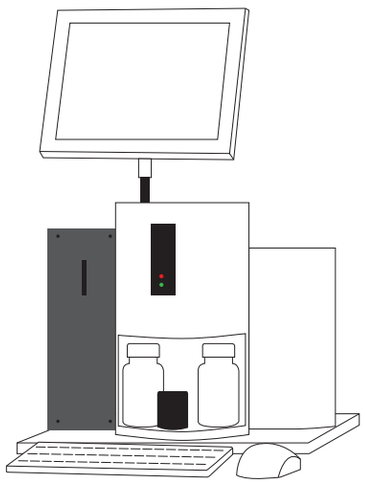
Techniques using Coulter Principle

CASY Counter

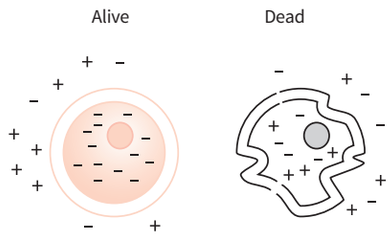
A CASY counter relies on the cell's membrane integrity and its impact on a cell's impedance to distinguish between living and dead cells. A cell's electrical impedance, as described by the Coulter Principle, can be measured with a tube in which cells are submitted to a low intensity current[14,15]. A software that distinguishes between the impedance of living and dead cells processes variations of the recorded impedance and transforms electric current into digital data. Just like the previously presented technique, a CASY counter can get the sample from manual or on-line sampling.

CASY counter acquisition is very precise but lacks accuracy as it only quantifies dead cells that are well advanced in the death pathway. The analysis cannot benefit from machine learning. Sampling can be either manual or automated (thus lacking or increasing accuracy and precision). As they are submitted to an electric current, cells cannot be returned to the culture. Therefore, the sample is wasted.

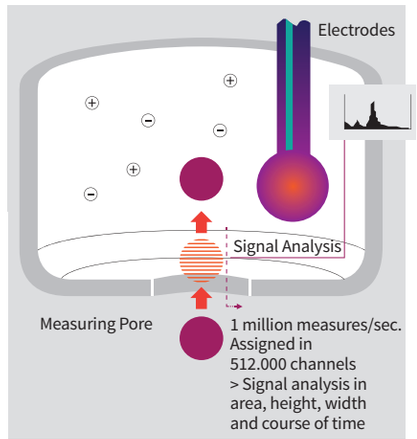
Sampling
Manual/On-line



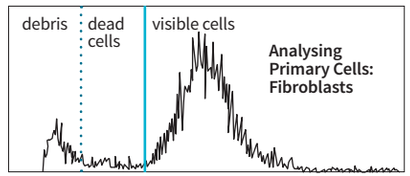
Principle
Integrity of the Membrane



Acquisition
Coulter Principle



Analysis Specific Software Analyzing Raw Data



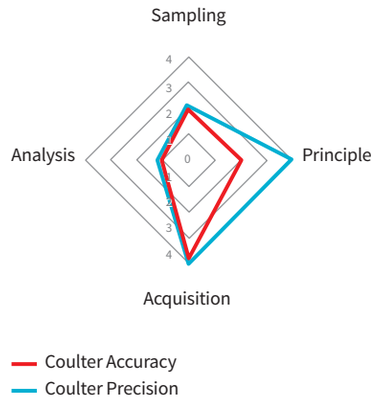


Figure VI: CASY Counter

Flow Cytometer

Flow cytometers[16] characterize dead cells by monitoring their membrane integrity and/or by labelling. Based on light scattering or the Coulter principle (but using a laser rather than electricity), they can study cells with a label based or label-free method (which can affect the preparation time of the sample) and sort cells according to parameters determined by the user. The cells go from a tube through a capillary (fluidics system) and are crossed by one or several laser beam(s) (optics system)[17]. The fluorescence features will not be explained here but the basic optics system can be described as two detectors:

- one for the Forward-SCattered light (FSC) that detects the light from the laser that passes around the cell, hence giving information about the size of the cell
- one for the Side-SCattered light (SSC) that detects the light reflected by the inner compounds or the cell external granularity.

After excitation of the samples by a laser beam(s), data is processed by a specific software (Electronics System)[17] transforming electric current into data. In order to extract information from the raw data, the user will have to configure some settings:

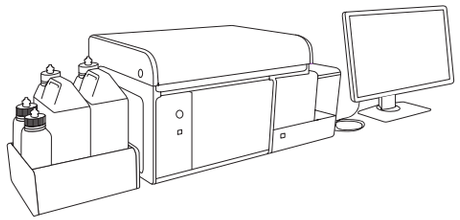
- Compensations: the setting used to prevent the interference between lasers
- Thresholds: the value from which a cell will be classified in one or another category
- Gates: the sets of cells that will be analyzed and/or taken into account for the final results
- Cell sorting: the system that enables the sorting of cells.

The electronics system is, with the sample preparation time, the weakest spot of this technique as it is heavily user-dependent with low reproducibility. Flow Cytometers can get samples in two different ways: manual or on-line sampling.

Flow cytometry is one of the most common solutions in labs, especially for research purposes. Data acquired can show many different things depending on the optional labelling of cells. However, it is time-consuming in terms of workload and demonstrates high variability between measurements as it suffers in precision and accuracy from manual analysis and sampling. Sampling can be automated but as cells are mixed with the buffer, they cannot be returned to the culture. The sample is wasted.

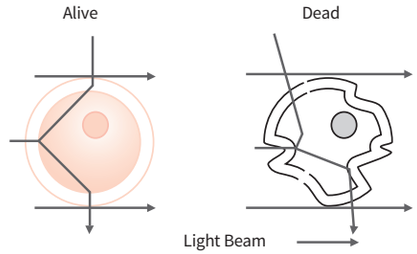
Sampling

Manual/On-line



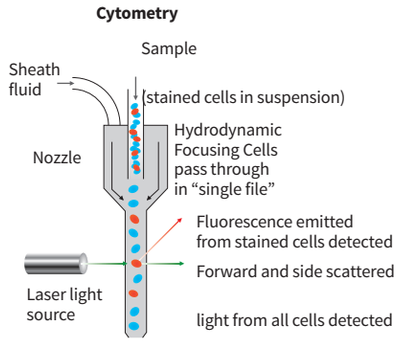
Principle

Integrity of the Membrane



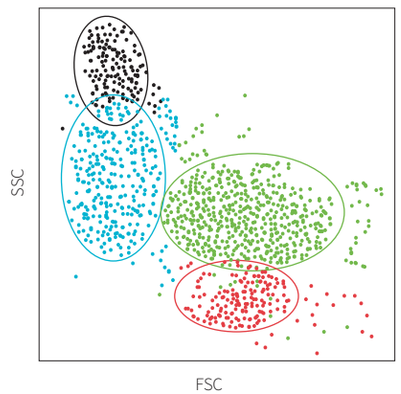
Acquisition

Cytometer Principle



Analysis

Specific Software Analyzing Raw Data



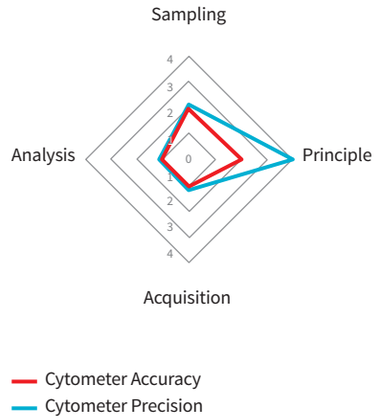


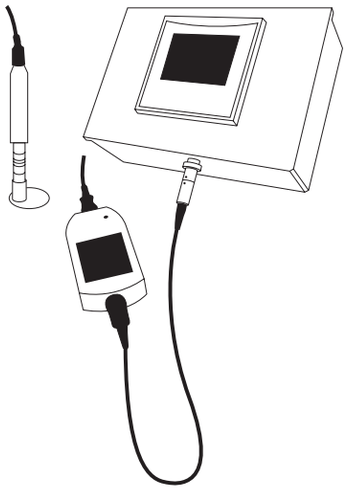
Figure VI : Flow Cytometer

Dielectric Spectroscopy Probe

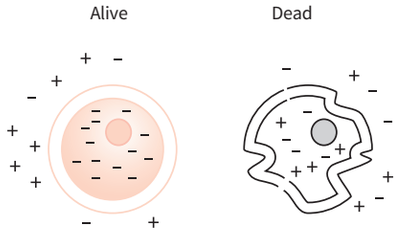
A dielectric spectroscopy probe relies on cell membrane integrity and its impact on the cells' impedance. A cell's electrical impedance, as described by the Coulter Principle, is directly measured in the cell culture where the impedance is correlated to the number of living cells in the medium[14,15]. An alternating electrical field is generated by the probe and the polarization and depolarization of the cells is measured. As viable cells behave like small capacitors the signal can be correlated to the bio-volume, giving the viable cell density. The polarization in the alternating electrical field only happens to cells with an intact membrane and thus viable cells as dead cells have leaky membranes and can't store a charge.[18,19,20] The probe is in the culture and therefore requires no sampling.

A dielectric spectroscopy probe is certainly one of the easiest solutions to implement. It does not need any sampling and it acquires data directly from within the culture medium. However, its analysis software cannot benefit from machine learning and the probe can only detect biomass.

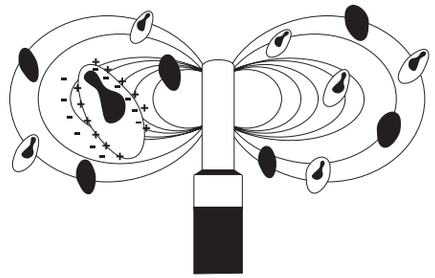
Sampling
In-line

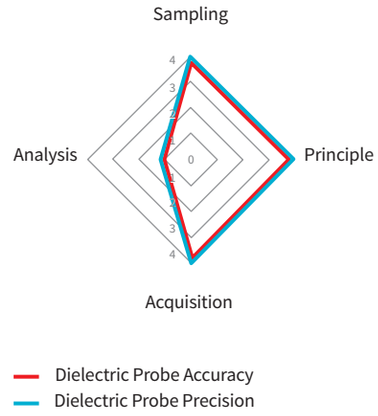


Principle
Integrity of the Membrane



Acquisition
Coulter Principle





Analysis
Specific Software Analyzing Raw Data

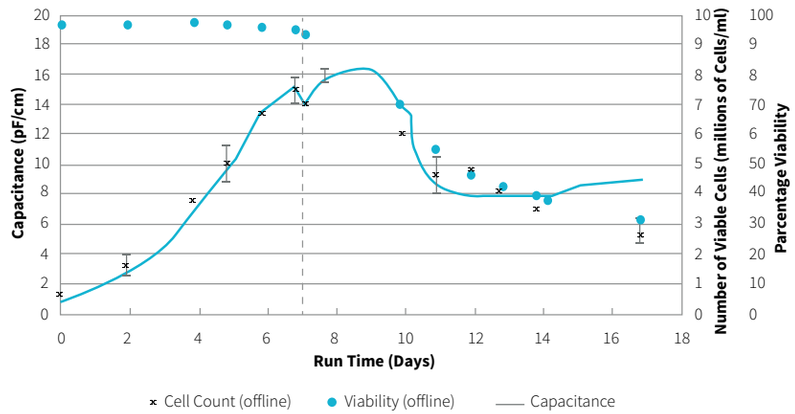


Figure VIII: Dielectric Spectroscopy Probe

Techniques using microscopy without staining

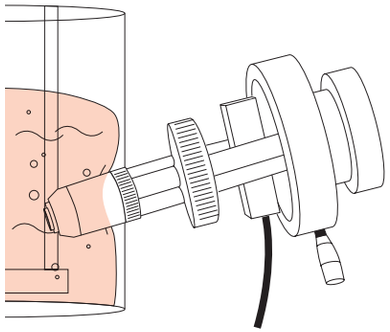
In Situ Microscope

An in-situ microscope[21] is used to study a cell's profile. It captures images of the whole culture, using red LED light to avoid disturbing the cells. The pictures are sent to a computer. The IRS directly works on the pictures/film. As the microscope is directly plunged into the culture, it needs no sampling.

An in-situ microscope does not need any sampling, acquires data directly from the culture medium and its analysis can benefit from machine learning. However, it is based only on cell profiles and so can lack precision depending on what death pathway the cells use.

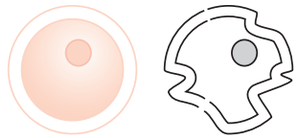
Sampling

In-line



Principle

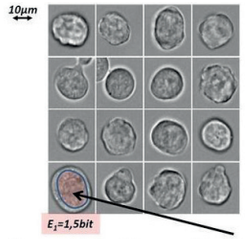
Cell Profiles



Acquisition

Image-Capture/Cell Profiles

Hybridoma-culture with high viability (appr. 96%)



Measure of texture inhomogeneity:

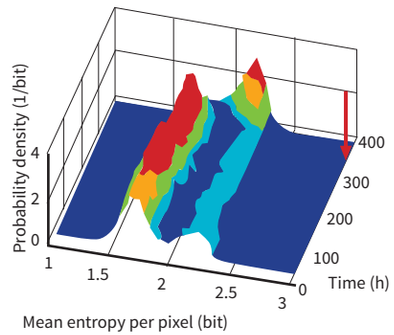
Hybridoma cells, a) entropy histogram, b) viability plot, osmotic shock at 230h

Entropy

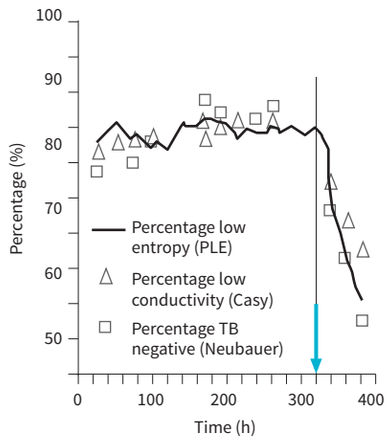
Analysis

Image Recognition Software

Wiedermann et al. (2011) Biotechnol bioeno 108, 2884-2993



Mean entropy per pixel (bit)



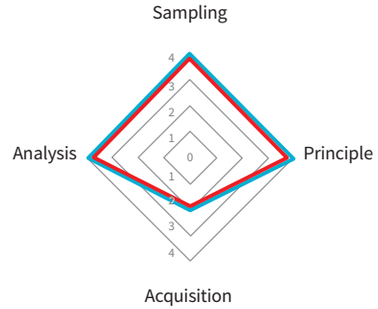


Figure IX: In Situ Microscope

Double Differential Digital Holographic Microscope

A double differential digital holographic microscope studies membrane integrity and cell profiles to distinguish between life and death. A dead cell with porous membrane diffuses light, whereas a living cell concentrates it in a light cone. Living and dead cells have thus different impacts on the light. By measuring this impact, a holographic fingerprint (based on 59 parameters) is captured. The OPL-analyzing software computes cell counting and viability based on this holographic approach. It acquires data in shades of grey and converts them into a colorized image. One of its best features is its ability to gather information about every single cell it visualizes. The microscope brings cells into its field of vision and returns them to the culture without disturbing them. Therefore, no samples are wasted.

A double differential digital holographic microscope has strong accuracy and precision as it is based on both membrane integrity and cell profiles. Acquired data can show many different things without labelling and the analysis software benefits from machine learning. The sampling is automated. As cells are not mixed with anything, they can be returned to the culture. Therefore, the sample is not wasted.

Sampling
In-line

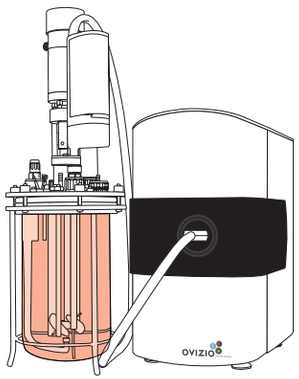
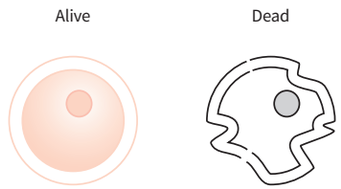
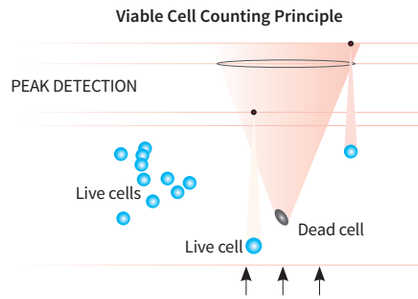


Figure X: D3 Holographic Microscope

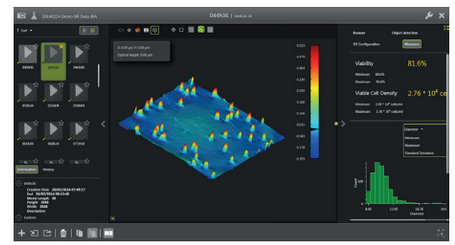
Principle
Integrity of the Membrane/Cell profiles

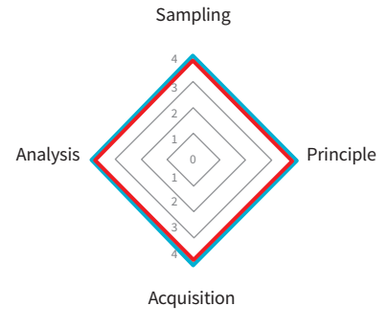


Acquisition
Differential Digital Holographic Microscopy



Analysis
OPL-analyzing algorithms





OVIZIO IMAGING SYSTEMS

The future of cell culture monitoring

We introduced the techniques for cell viability monitoring and the way they perform. We took the option of evaluating them by focusing on their accuracy and precision. While many other criteria could have been chosen (such as price, volume…), those two seemed more relevant in a context in which the main demand is for reproducibility and measurement as close to reality as possible.

The table below compares each different method described above.

Techniques	Principle	Acquisition	Analysis	Machine Learning	Sampling	Approach	User-dependent
Hemocytometer	Integrity of the Membrane	Image Capture - Colorimetry	Manual	No	Manual	Invasive	Yes
Image Mosaïc	Integrity of the Membrane	Image Capture - Colorimetry	Image Recognition Software	Yes	Manual	Invasive	Yes
Cell Imaging System	Integrity of the Membrane/Cells Profiles	Image Capture - Colorimetry/Cells Profiles	Image Recognition Software	Yes	Manual/On-Line	Invasive	Yes
Flow Cytometer	Integrity of the Membrane/Labelling	Coulter Principle	Specific Software Analyzing Raw Data-Manual Settings	No	Manual/On-Line	Invasive	Yes
CASY Counter	Integrity of the Membrane	Coulter Principle	Specific Software Analyzing Raw Data	No	Manual/On-Line	Invasive	Yes
In Situ Microscope	Cell Profiles	Image Capture - Cells Profiles	Image Recognition Software	Yes	In-line	Non-Invasive	No
Dielectric Spectroscopy Probe	Integrity of the Membrane	Coulter Principle	Specific Software Analyzing Raw Data	No	In-line	Non-Invasive	No
D3 Holographic Microscope	Integrity of the Membrane	Differential Digital Holographic Microscopy	OPL-analyzing algorithms	Yes	In-line	Non-Invasive	No

accurate/precise

inaccurate and/or imprecise

Table I: Summary Table

By crossing this data, the graph below can be drawn. It compares the accuracy and precision of the different techniques.As we can see, a shift to automated techniques grows when accuracy and precision are required. The newest tools tend to be increasingly automated and user-independent, no matter which method is used: Trypan Blue, Coulter Principle, Holography or Cell Profiles.

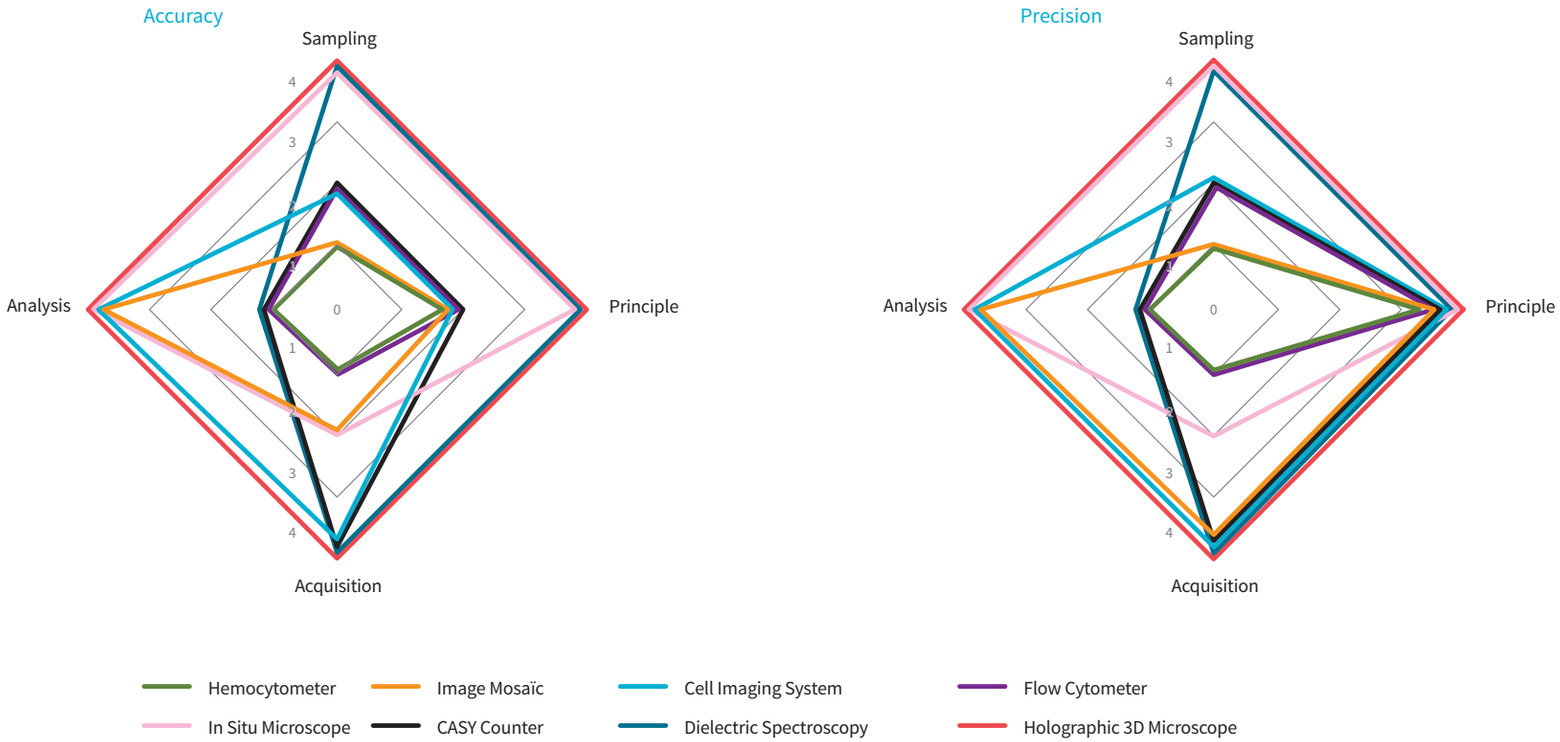


Figure XI: Chart comparing the different techniques according to their accuracy and precision

This tendency towards automation has multiple aims. It eliminates any risk during sampling, which is critical especially in production zones. It can also standardize the sampling and bring more homogenous samples. It generates more comparable and reproducible results. As it reduces the impact of humans, it deletes multiple causes of errors and subjectivity. It speeds up the results and the response (the difference is obvious for flow cytometry with automated sampling). This enables parameters of the culture environment to be corrected more rapidly. It reduces the need for an operator, which gives them extra time to focus on other tasks

Apart from this trend, the four systems still share the viability monitoring market. This mainly comes from the fact that different processes require different elements. For example, a process that does not need a precise measurement of viability but has to assess the expression of a gene of interest will likely be monitored by a flow cytometer. Another example is the use of hemocytometers in small labs, schools or processes that do not need efficient yields. Nevertheless, even if they coexist, they do not distinguish between life and death at the same step.

But there are countless other parameters that can be checked. Whereas early in the cell culture history, the main concerns were oxygenation and pH, cell viability is now preeminent. So, what are the next key parameters?

It is most likely that it will be a combination of multiple factors. The emergence of machine learning and big data analysis will enable us to consider parameters that we do not yet consider as crucial to build a model closer to reality and deliver the best possible environment for the expected results. With these new analysis tools, future techniques will not suffer from restrictions from the amount or variety of data they will acquire. Altogether, this will lead to a more detailed knowledge of the culture and a better adjustment to the cell's environment. Another important feature these devices will need is flexibility with respect to the volume of the culture being monitored. This will enable a quick scaling-up of the process.

Hence, future techniques must be accurate, precise, flexible and multi-factorial to meet those new requirements.

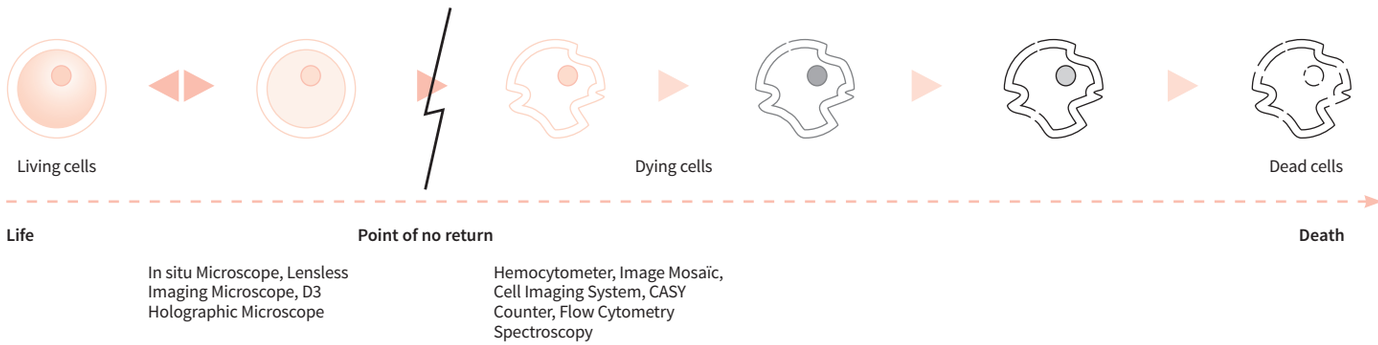


Figure XII: Cell Death according to each technique

References

1. Guidance for Industry and Review Staff, PAT — A Framework for Innovative Pharmaceutical Manufacturing and Quality Assurance, Rockville, FDA, August 2003, https://www.fda.gov/ohrms/dockets/ac/04/briefing/2004-4052B1_15_GFI-Draft-PAT-Guidance.pdf

2. MIRANDA MDOS S, BRESSAN FF, DE BEM TH, MERIGHE GK, OHASHI OM, KING WA, MEIRELLES FV, Nuclear transfer with apoptotic bovine fibroblasts: can programmed cell death be reprogrammed?, Cell Reprogram. 2012 Jun;14(3):217-24. doi: 10.1089/cell.2011.0080.

3. LEBER B, LIN J, ANDREWS D, Embedded together: The life and death consequences of interaction of the Bcl-2 family with membranes, Apoptosis, Volume 12 (5) – May 1, 2007

4. VAN DOORN WG, Plant programmed cell death and the point of no return, Trends Plant Sci. 2005 Oct;10(10):478-83.

5. ELMORE S, Apoptosis: A Review of Programmed Cell Death, 2007, Toxicologic Pathology, P 495-516, V 35.

6. TRAN SL, PUHAR A, NGO-CAMUS M, RAMARAO N, Trypan Blue Dye Enters Viable Cells Incubated with the Pore-Forming Toxin HlyII of Bacillus cereus, PLoS One. 2011; 6(9): e22876.Published online 2011 Sep 6. doi: 10.1371/journal.pone.0022876 PMCID: PMC3167804

7. PICCININIA F, TESEIB A, PAGANELLIB G, ZOLIB W, BEVILACQUA A, Improving reliability of live/dead cell counting through automated image mosaicking, computer methods and programs in biomedicine 117 (2014) 448–463

8. ALTERMATT F, FRONHOFER EA, GARNIER A, GIOMETTO A, HAMMES F, KLECKA J, LEGRAND D, MÄCHLER E, MASSIE TM, PENNEKAMP F, PLEBANI M, PONTARP M, SCHTICKZELLE N, THUILLIER V & PETCHEY OL, Big answers from small worlds: a user's guide for protist microcosms as a model system in ecology and evolution, Supplementary information for ALTERMATT et al. Methods in Ecology and Evolution. DOI: 10.1111/2041-210X.12312

9. JULIUS MH, MASUDA T, HERZENBERG LA (1972). "Demonstration that antigen-binding cells are precursors of antibody-producing cells after purification with a fluorescence-activated cell sorter". Proc. Natl. Acad. Sci. U.S.A. 69 (7): 1934–8.

10. "FACS MultiSET System" (PDF). Becton Dickinson. Archived from the original (PDF) on October 18, 2006.

11. JAVORNICKÝ P, Revise některých metod pro zijšťováni kvantity plankton (Revised method for quantitative enumeration of phytoplankton), Prague, 1958 Sci. Pap. Inst. Chem. Technol. Fuel and water 2 (part1)

12. SHAMIR L, DELANEY JD, ORLOV N, ECKLEY DM, and GOLDBERG IG, Pattern Recognition Software and Techniques for Biological Image Analysis, PLoS Comput Biol. 2010 Nov; 6(11): e1000974. Published online 2010 Nov 24. doi: 10.1371/journal.pcbi.1000974 PMCID: PMC2991255

13. BRINKMANN M, LÜTKEMEYER D, GUDERMANN F, LEHMANN J, New technologies for automated cell counting based on optical image analysis ;The Cellscreen, Cytotechnology. 2002 Jan;38(1-3):119-27. doi: 10.1023/A:1021118501866.

14. JUSTICEA C, BRIXB A, FREIMARKA D, KRAUMEC M, PFROMMB P, EICHENMUELLERD B, CZERMAK P, Process control in cell culture technology using dielectric spectroscopy, Biotechnology Advances Volume 29, Issue 4, July–August 2011, Pages 391–401

15. PATELA P, MARKX GH, Dielectric measurement of cell death, Enzyme and Microbial Technology Volume 43, Issue 7, 10 December 2008, Pages 463–47

16. ABU-ABSI NR, ZAMAMIRI A, KACMAR J, BALOGH SJ, SRIENC F, Automated Flow Cytometry for Acquisition of Time-Dependent Population Data, Cytometry part A 51A:87-96 (2003)

17. BD Biosciences, Introduction to Flow Cytometry: A Learning Guide, Manual Part Number: 1-11032-01, April 2000

18. Collective work, Incyte - Viable Cell Density, https://www.hamiltoncompany.com/products/process-analytics/sensors/cell-density/incyte-viable-cell-density, 2017

19. Collective work, On-line measurement of cell vitality, https://www.chromservis.eu/i/on-line-measurement-of-cell-vitality, 2017

20. POGGENDORF I, LÜTHI R, FRANK M, McNEEL K, Cell Viability in Real Time Online Viable Cell Density Measurements on Microcarriers Caspar Demuth, June 15, 2016 (Vol. 36, No. 12)

21. WIEDEMANN P; GUEZ JS; WIEGEMANN HB; EGNER F; QUINTANA JC; ASANZA-MALDONADO D; FILIPAKI M; WILKESMAN J; SCHWIEBERT C; CASSAR JP; DHULSTER P; SUHR H, In situ microscopic cytometry enables noninvasive viability assessment of animal cells by measuring entropy states, Biotechnology and Bioengineering, Volume 108 (12) – Dec 1, 2011