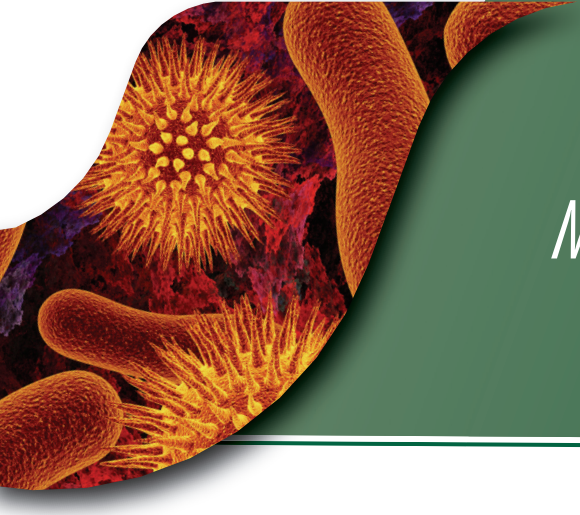

New Technologies for More Powerful Cell Analyses

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Changes in morphology, interactions with other cells and the environment, complex signalling pathways, and the localisation of events are just some of what researchers explore in the quest to identify targets for drug development efforts. Our knowledge of the inner workings of cells and their interactions is advancing rapidly, supported by technologies that simplify the acquisition of data and deliver more sophisticated data on cell populations and individual cells.

These advances derived from a range of innovations: improvements on decades-old manual techniques, miniaturised technology, multiplexed analyses, and the integration of historically 'stand-alone' technologies to deliver greater insights.

In this article we explore new approaches to monitoring cell health, the cell cycle, and apoptosis, powerful multiplexed assays that provide a more holistic view of the cell, and immune function assays based on a unique combination of flow cytometry and imaging.

Advancing the Assessment of Cell Health

Cell counting is a necessary step when seeding and passaging cells or when preparing experiments for cell-based assays. Cell counts can also be used for monitoring the health of cultures, rates of proliferation, and assessing immortalisation for transformation.

Despite the need for speed and accuracy, the vast majority of cell counting is accomplished manually with a hemocytometer – a technique first introduced in the late 1800's for counting blood cells. Researchers can also count cells using automated counting instruments such as vision-based counters, flow cytometers, or systems that incorporate the Coulter principle. Coulter technology, first developed in the 1940's to count blood cells, is used in the majority of automated cell counters.

Although automated cell counting systems overcome the challenges of manual counting with a hemocytometer, the cost of these benchtop instruments can be prohibitive.

The latest innovation in cell counting combines the ease of automated instrumentation and the accuracy of Coulter technology in hand-held format. Beyond simply being a means to count cells, this technology provides immediate insights into the health of cell populations.

The Scepter™ cell counter (Merck Millipore) incorporates Coulter impedance-based particle detection in a miniaturised format. The instrument, which is the size of an automated pipette, incorporates a combination of analogue and digital hardware for sensing, signal processing, data storage, and graphical display. The disposable tip is engineered with a microfabricated, cell sensing zone that enables discrimination by cell size and cell volume at sub-micron and sub-picolitre resolution.

Cell population statistics are displayed as a histogram directly on the instrument. Data can be used to gain immediate insights into the health of cell populations. Well-maintained cultures will show a relatively high quantity of cells with the expected diameter.

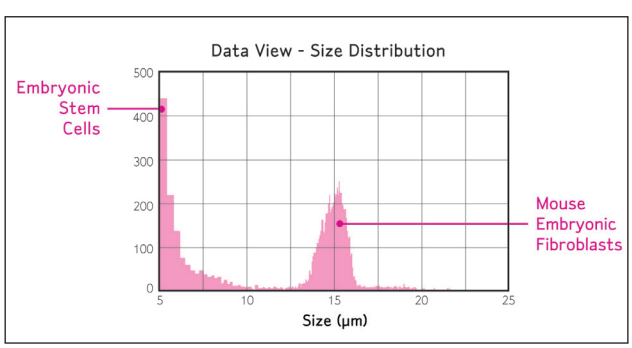


Figure 1. Histogram showing embryonic stem cells and fibroblast cells from the same culture. (Data courtesy of Qi-long Ying, PhD, Eli and Edythe Broad Center for Regenerative Medicine and Stem Cell Research at the University of Southern California)

In addition to automated cell counts, this technology can be used to advance the understanding of the mechanisms underlying embryonic stem cell self-renewal and differentiation. Figure 1 shows a histogram of embryonic stem cells grown on a fibroblast feeder layer; divergent peaks are used to specifically count one population of cells or another in a single sample. Clinically-relevant cell types can also be monitored and quantified by tracking the differentiation of embryonic stem cells. Significant shifts in the histogram occur as stem cells differentiate, allowing researchers to monitor dynamic changes.

More Powerful, More Compact

Despite the advantages of cytometry, the size, complexity, cost, and maintenance requirements of conventional systems have historically

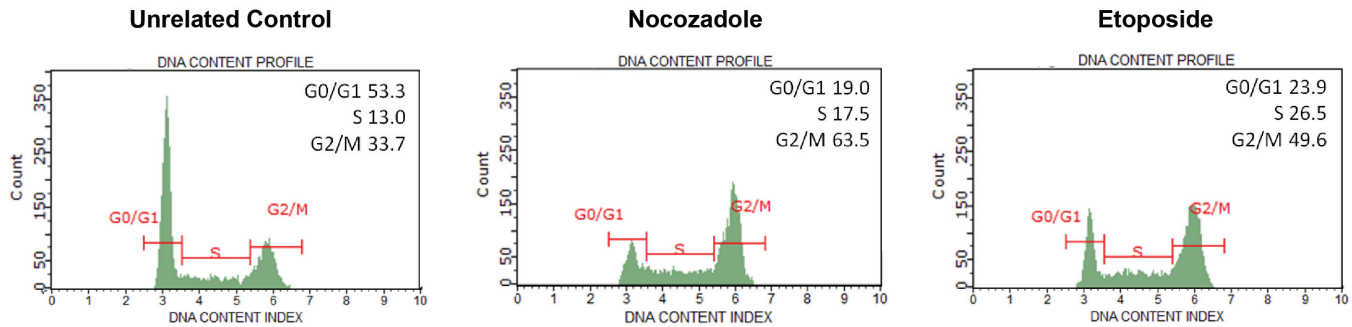


Figure 2. Impact of cell cycle disrupting compounds on Jurkat cells analysed using the Muse™ Cell Cycle Assay. Nocodazole, a microtubule disrupter, leads to cell cycle arrest in G2M phase; Etoposide, a known anti-cancer compound, also causes G2/M arrest.

confined their use to core facilities and large laboratories with expert users.

We are now able to miniaturise fluidics and optics for fluorescent detection (Muse™ cell analyser, Merck Millipore) to permit researchers to access the power of cytometry at their lab bench even if they don't have experience with the technique. Laser-based fluorescence detection of each cell event allows evaluation of three cellular parameters.

Cell Cycle Analysis

The cell cycle represents one of the most fundamental processes in eukaryotic cells, resulting in cell growth and division into two daughter cells. The regulation of cell cycle is critical to cell survival, as it governs the repair of genetic damage and the prevention of uncontrolled cell division. Defects in cell cycle regulation are a characteristic feature of tumour cells, and mutations in the genes involved in controlling the cell cycle are extremely common in cancer. Cell cycle analysis has become increasingly important in the understanding of action of anticancer compounds or studying mechanisms of cell division.

The analyser allows for rapid, quantitative measurements of the percentage of cells in the G0/G1, S, and G2/M phases of cell cycle. The assay simplifies an analysis that has traditionally required complicated instrumentation and training, and allows for users to obtain information on cell cycle distribution right at the bench.

The nuclear DNA stain propidium iodide (PI) is used to discriminate cells at different stages of the cell cycle, which differ in DNA content. Resting cells (G0/G1) contain two copies of each chromosome. As cells begin cycling, they synthesise chromosomal DNA (S phase). Fluorescence intensity from the DNA intercalating dye, PI, increases until all chromosomal DNA has doubled (G2/M phase). At this state, the G2/M cells fluoresce with twice the intensity of the G0/G1 population. The G2/M cells eventually divide into two cells. The assay thus utilises the differential staining of cells based on DNA content. Figure 2 shows the effects of a variety of conditions on the cell cycle.

Apoptosis Detection

Apoptosis, or programmed cell death, is an important regulator of cell growth and proliferation. Induction of apoptosis is characterised by a progressive series of cellular biochemical and morphological changes. One of the hallmarks of apoptosis is the translocation of phosphotidylserine from the inner to the outer leaflet of the plasma membrane and exposure to the outer surface of the cell. This universal phenomenon is independent of species, cell type, and induction system and occurs early in the apoptotic process. Figure 3 shows the impact of apoptosis-inducing compounds on two different cell lines using the analyser.

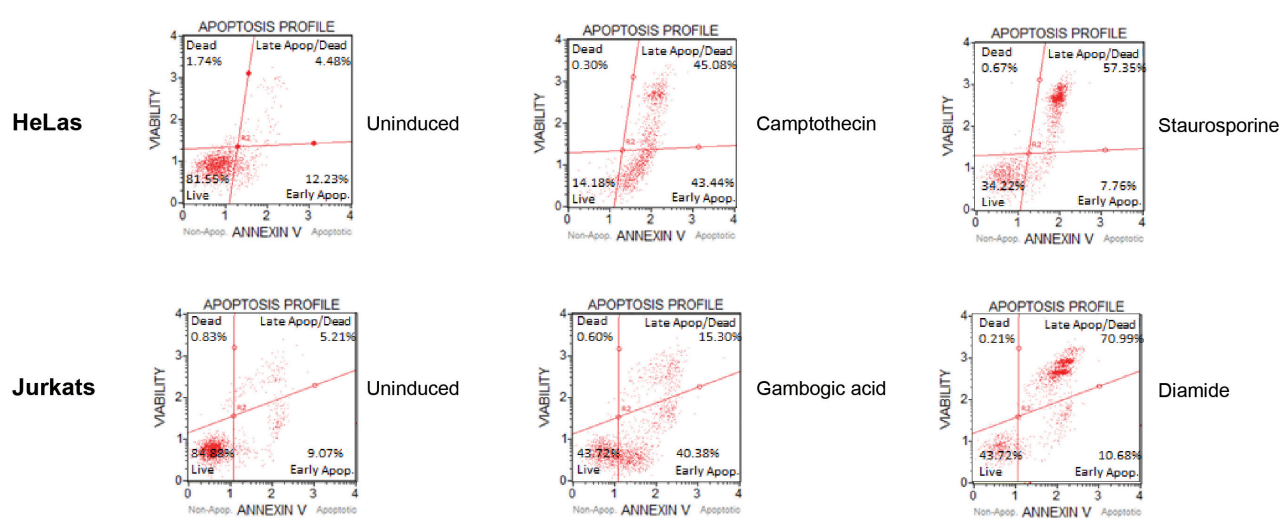
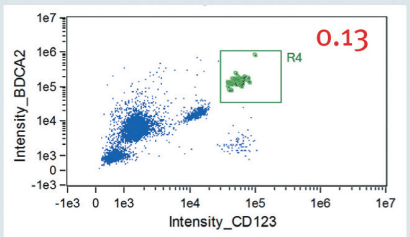
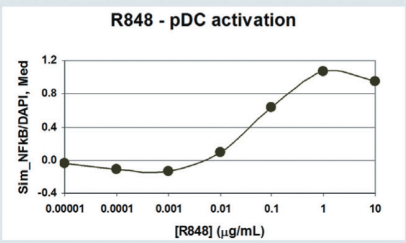


Figure 3. Impact of apoptosis-inducing compounds on HeLa cells (adherent lines) and Jurkat cells (suspension lines) analysed using the Muse™ Annexin V & Dead Cell Assay.





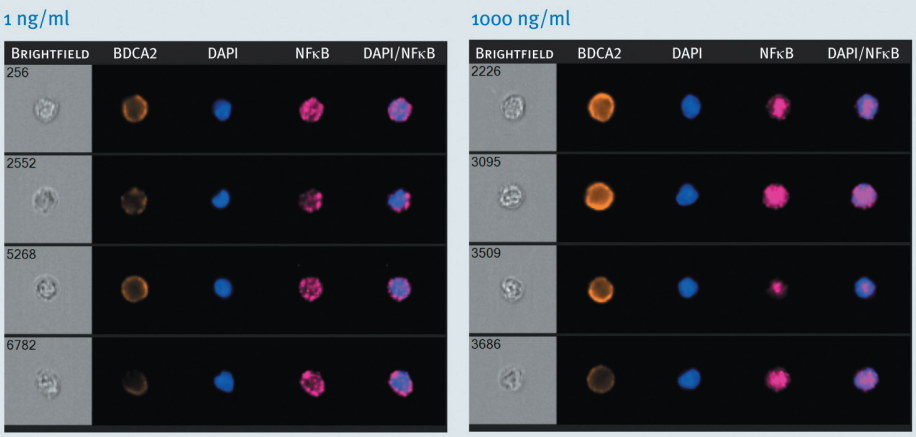


Figure 4. Measurement of NF-κB activation in whole blood pDC.

The assay is based on the detection of phosphatidylserine (PS) on the surface of apoptotic cells, using fluorescently labeled Annexin V in combination with a dead cell marker, 7AAD. Annexin V is a Ca2+-dependent phospholipidbinding protein that has a high affinity for PS, a membrane component normally localised to the internal face of the cell membrane. Early in the apoptotic pathway, molecules of PS are translocated to the outer surface of the cell membrane where Annexin V can readily bind to them. Late-stage apoptotic cells show loss of membrane integrity. The membrane impermeant dye 7-ADD is used to distinguish dead cells from early apoptotic cells.

The assay can thus distinguish four populations:

- Viable cells, not undergoing detectable apoptosis: Annexin V (–) and dead cell marker (–)
- Early apoptotic and dead cells: Annexin V (+) and dead cell marker (–)
- Late apoptotic or dead cells: Annexin V (+) and dead cell marker (+)
- Cells that have died through non-apoptotic pathway: Annexin V (–) and dead cell marker (+)

Integrating Flow Cytometry and Imaging

Many assays for immune function require imaging, but immune cells present significant challenges to image-based analysis due to their rarity and the need for simultaneous multispectral immunophenotyping, making statistically robust quantification difficult. Thus, immune function assays are ideally performed by a technology platform that combines flow cytometry and imaging (ImageStream®, Merck Millipore), which quantifies imagery of large populations of cells.

Following is an example of one immune function assay, activation of NF-κB translocation.

As shown in Figure 4, pDC express pattern recognition receptors that transmit activating signals upon ligand binding. Translocation of NF-κB was measured as a marker for TLR7-induced activation in whole blood pDC. NF-κB translocation was measured using the Similarity score (J Immunol Methods 311:117) for the gated pDC from whole blood samples exposed to a range of R848 doses. Images of representative cells from the 1ng/ml (left) and the 1000ng/ml (right) samples are shown.

Multiplexing for More Information

The study of individual biomarkers is often inadequate to fully characterise processes in both normal and diseased states. Multiplexing of analytes offers significant advantages when studying complex pathways and biomarker interactions such as those found in angiogenesis.

Angiogenesis, the formation of new blood vessels, is a tightly controlled process with a key role in normal growth, development, wound healing and transplantation rejection. When angiogenic pathways are disrupted, insufficient or excessive angiogenesis results in diseases such as coronary artery disease, ischemic chronic wound healing, autoimmune disease, macular degeneration and cancer. Angiogenic signalling in tumours is similar to normal angiogenesis, mediated by soluble growth factors, membrane-bound receptors, and cell-cell and cell-matrix interactions.

Traditional 'singleplex' protein detection methods can be impractical for efficient exploration of complex cell signalling pathways and protein-protein interactions such as those found in angiogenesis. The xMAP multiplex platform (Luminex) enables researchers to differentiate dozens of analytes per sample, significantly reducing the time, labour and costs associated with methods such as ELISA and Western blotting.

The xMAP platform consists of fluorescently dye-labelled magnetic microspheres, a flow cytometry-based (LX200™ or FLEXMAP 3D®) or CCD camera-based (MAGPIX®) instrument, and software for data acquisition

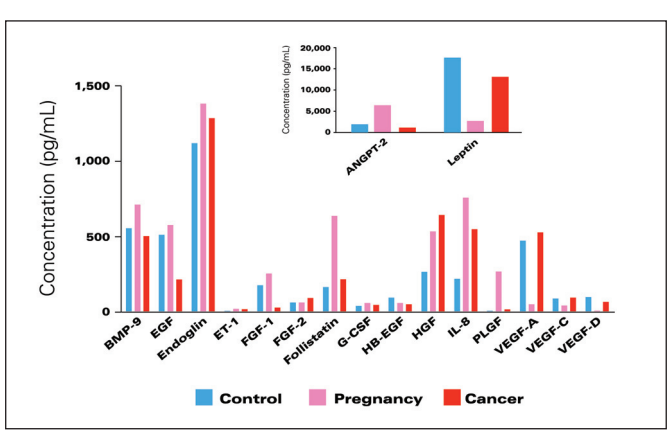


Figure 5. Serum sample results run using the multiplexed human angiogenesis / growth factor panel.

and analysis. With the xMAP platform, sandwich assays are performed on a bead. Each bead contains two red dyes; ten different concentrations of the dye yield 100 different possible combinations. Since the beads are in solution rather than fixed to a plate, up to 50 different beads with different capture antibodies can be used with one sample yielding results for up to 50 different proteins. When the beads pass through the reader, or in the case of MAGPIX camera, flow into the chamber, the ratio of the two dyes indicates the bead number.

Streptavidin-phycoerythrin, which fluoresces green, is used as the common detection reagent binding to biotinylated antibodies. The detection system reads red and green thus providing identification of the analyte and quantitation of the amount bound to the bead.

The MILLIPEX® MAP Angiogenesis panel (Merck Millipore) contains seventeen analytes reported to be involved in the process of angiogenesis and is used in combination with the Luminex system. The configurable panel represents pro-angiogenic and anti-angiogenic factors, allowing researchers to study the process of angiogenesis in both normal and diseased states.

A collection of serum samples obtained commercially (Bioreclamation) was analysed using the angiogenesis panel (Figure 5). The mean value observed from healthy control (n = 8), pregnancy (n=5) and various tumour types (n= 35) is displayed. The insert shows two analytes (leptin and angiopoietin-2) which have higher values in serum than the other fifteen members of this panel.

Perhaps the biggest challenge facing scientists today is navigating biology's incredible complexity and applying these insights to clinical medicine. Both basic research and drug discovery efforts continue to advance with the help of powerful new tools and technologies. These approaches enable the study of large cell populations and individual cells and help us understand the changing dynamics within complex biological networks that define healthy and disease states.