Addressing the new requirements of cellular research with continuous live-cell analysis

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In his classic futuristic science-fiction novel ‘1984’, George Orwell described a world of omnipresent covert government surveillance and monitoring of the mass population. Big Brother saw everything. Information was knowledge and power.

Thirty years ‘on’, but not quite to this extreme, huge swathes of information are now acquired and managed by non-invasive monitoring systems. Whether it’s high street security cameras, wearable technology for the health conscious or activity tracking on your IT server, continuous monitoring has become a part of everyday life.

So, how is this relevant to biomedical research on cells?
The Changing Landscape of Cellular Research

Exciting recent developments in continuous live-cell monitoring techniques are now re-defining the ways that we learn from and use cells in the laboratory. These technologies capture valuable, decision making cellular data over time in ways, and at a scale, that was previously impossible. Importantly, the attributes of these solutions are closely aligned with significant changes in the overall landscape of cellular research.

In the search for greater relevance, researchers are shifting away from simple, recombinant cell systems to primary cells and stem-cell derived cells that are often human and patient-specific. Advanced in vitro co-culture and tissue organoid models are becoming increasingly common. Whilst target-specific, reductionist assays still remain important (and will likely play a role for many years to come), phenotypic, cell-based assays are now becoming more popular as they allow holistic, integrated cell functions to be measured. While these changes present unique opportunities for innovative research, they also bring fresh technical challenges and new requirements for cellular workflows. This article outlines how advances in continuous live-cell monitoring and analysis methods are addressing these changing needs.

Immortalised cell lines

Nowadays, the husbandry of classic cell lines such as CHO, HEK and HELA cells is straightforward and routine. Advances in molecular techniques allow scientists to easily express or manipulate genes in these cells, even for constructs encoding large and complex proteins. Stable gene expression can be induced for many cell passages using kit-based, turnkey methods. Typically, recombinant cells are robust and predictable; they grow quickly, homogeneously and consistently for long periods of time with few complexities introduced by differentiation or loss of expression. Overall, they are low-cost and high-yield, so researchers have the luxury of discarding unused ‘spare’ cells on a regular basis.

Primary cells

Compared to immortalised cell lines, primary cells are more likely to accurately reflect how biological systems function in vivo. However, primary cells are generally more difficult to obtain in large numbers and many do not divide, so they cannot be further up-scaled (e.g. cardio-myocytes and neurons). Some primary cell types do not survive for long periods in culture (e.g. human neutrophils) or may rapidly de-differentiate (e.g. human hepatocytes). Lastly, the genetic manipulation of primary cells is more challenging than for immortalised cells and may require special constructs and transfection procedures (which are often detrimental to cell health).

Stem cells

Stem cells offer greater flexibility than both primary and immortalised cells. The technology promises a world where fully differentiated mature cells of any type can be obtained from replicating stem cell pre-cursors. These cells
also offer a number of diagnostic and therapeutic applications, as the properties of cells derived from different patients can be compared to help dissect genetic pre-disposition and the aetiology of disease. Mesenchymal stem cells and differentiated cells can also be considered as therapies per se. Combining these approaches with the latest developments in precision gene editing (e.g. CRISPR/Cas9) brings the idea of true personalised medicine ever nearer.

That being said, the protocols for reprogramming, scaling and differentiating stem cells are currently lengthy, technically challenging and somewhat poorly defined at the mechanistic level. To overcome these difficulties, a number of commercial suppliers now offer cryopreserved, differentiated cells. However, these are prohibitively expensive for many labs to use routinely and at scale. The properties of stem cells also change over time as they differentiate and mature, and in many cases the final yield of healthy, fully differentiated cells is low. This dynamic nature of stem-cell derived and primary cell cultures places greater emphasis on the need for active management and real-time decision making by the scientist, especially when compared to immortalised cells.

**The role of the microenvironment**

The true extent to which the microenvironment affects cell behaviour is also emerging. In an effort to reproduce true physiology and pathology, researchers are devising more elaborate co-culture and multi-culture cell systems. As such, the growing of cells in bio-matrices and scaffolds to form 3-dimensional organoids and microtissues is increasing in popularity, as are ‘organ on a chip’ systems. These experiments can be difficult and require significant optimisation, placing new burdens on the detection systems used for quality control and analysis of the cells of interest.

Overall, cellular models are fundamentally changing. The new cell systems that are now available may prove more relevant and translational, but they are also more complex and costly. Using cutting-edge cellular models, experimental studies require increased time and effort. The cells used are precious and not to be wasted. They are also dynamic and must be carefully observed.

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### Benefits & Challenges of Different Cell Model Systems

<table>
<thead>
<tr>
<th>Model System</th>
<th>Relative Cost</th>
<th>Ease of use</th>
<th>Availability</th>
<th>Relevance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Host cell Lines e.g. CHO, HEK</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
<tr>
<td>Tumour Cell Lines e.g. MDA-MB231</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
<tr>
<td>Primary Cells e.g. human blood cells</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
<tr>
<td>Co-multi cultures e.g. neurones &amp; astrocytes</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
<tr>
<td>iPSC-Derived Cells e.g. cardiomyocytes</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
<tr>
<td>Microtissues/Organoids e.g. hepatic structures</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
<td>⬤</td>
</tr>
</tbody>
</table>

Comparison of the typical attributes of cell model systems – relative cost (1=low), ease of use , availability and perceived translational relevance.
Defining the Need - Continuous Live Cell Analysis

From the above, experimental methods that inform of changes in the properties of living cells over the long term are required to address this added dynamic complexity. Ideally this information would be gathered throughout the life of the cells and in the context of other cells and their microenvironment. The approach needs to recognise and report cell heterogeneity. For cost and time reasons, methods that extract the most decision-making information from the smallest numbers of cells are favoured. This could be achieved directly (e.g. by assay miniaturisation), or indirectly (e.g. by minimising the number of studies and repeat assays that are required). Other ways to increase efficiency could include reducing the number of assay failures and the amount of initial assay development work that is required.

Cell-health, reporter and phenotypic assays that provide real-time data and that do not perturb cells will allow researchers to better understand the timeline of biological processes and make informed decisions regarding their cell husbandry and analysis workflows. Critically, meeting these requirements should not be at the expense of ease-of-use and simplicity to the researcher. Fortunately, a number of techniques are emerging to meet these growing needs, as discussed below.

Non-invasive, live-cell analysis

There are two main emergent technologies for non-invasive, long-term cell analysis – electrical monitoring and live-cell imaging. Neither is especially new, but both have evolved in ways that are aligned to the changing needs of cell biologists.

(i) Electrical monitoring

For electrical monitoring, cells are plated on electrodes embedded in plastic and cellular impedance or extracellular field potentials are recorded over time. High frequency (kHz) data acquisition and sampling is possible, and by placing the system in a cell incubator, signals can be recorded for long periods of time (days) without disturbing the cells. The most compelling applications are related to the analysis of excitable cells. For example, changes in how human stem-cell derived cardiomyocytes beat and contract can be measured over days and weeks using impedance recordings, allowing researchers to study both acute and chronic cardio-toxicity during drug development. For neurons, multi-electrode array chips have proven useful for the long-term tracking of action potential spike patterns (e.g. when measuring neurogenesis and the development of neural networks). Impedance values also change as cells proliferate, migrate, spread, or adhere to the electrodes, forming the basis for a range of other phenotypic assays. Importantly, the work flows are simple and do not perturb the cells. On the down side, specialist consumables (electrode plates) and large numbers of cells are often required. The systems also provide little spatial context, so collecting information about where cells are located and how they are interacting is problematic. Finally, impedance can be considered a fairly blunt tool for analysing non-excitable cells; signal changes in these cells can arise from a wide range of different cellular functions, making data interpretation difficult.
(ii) Live-cell imaging
For many scientists, live-cell imaging conjures up visions of an elaborate inverted microscope system, complete with a bewildering array of heated stages, light-source options, complex image analysis software and more. Typically, a small number of cells are viewed in a dish or plate and observed for a few minutes (or hours at most). As such, the idea of using a microscope to track cells over several days or weeks is considered unlikely by most researchers, especially if the imaging system is housed in a core facility shared by other colleagues, who are also keen to use the system. These factors limit the scale and throughput offered by most live-cell imaging workflows.

Over the last few years, different systems have evolved to overcome these challenges, often by simplifying these workflows and taking the strain out of live-cell imaging. A number of inexpensive, compact, single-plate microscopes have been developed, specifically designed to fit inside standard cell incubators and built with convenience and affordability in mind. Compared to high-end imagers, the optics and functionality do tend to be somewhat limited. Nevertheless, they provide entry level access to live-cell analysis for non-expert users. The workflows are extremely simple – you just place your cells on the stage and ‘press go’. For these systems, the software tools are geared more toward cell monitoring than detailed phenotypic analysis. For example, it is possible to carry out remote viewing and to configure the system to trigger automated e-mail alerts when cells have reached a given confluence. Some systems offer basic fluorescence measurements. In most cases, only a single plate can be analysed at a time and the software is not able to perform sophisticated image analysis.

At the other end of the spectrum, many high-content imaging devices and micro-plate imagers now have heated stages or chambers to accommodate living cells. Bright field optics for ‘label-free’ data capture have been added to their already impressive fluorescence capabilities, further extending the applications available. These solutions certainly provide high throughput and allow

Continuous Live-Cell Analysis: Imaging Solution Types

<table>
<thead>
<tr>
<th>Type</th>
<th>Environmental Control</th>
<th>Flexibility in Optics</th>
<th>Capacity, Throughput*</th>
<th>Automated Analysis*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compact Microscope</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
</tr>
<tr>
<td>e.g. Jull, CytoSmart</td>
<td>(In Incubator)</td>
<td></td>
<td>(1 plate/flask)</td>
<td></td>
</tr>
<tr>
<td>Live Cell Imager</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
</tr>
<tr>
<td>e.g. IncuCyte ZOOM</td>
<td>(In Incubator)</td>
<td></td>
<td>(6 plates/flasks)</td>
<td></td>
</tr>
<tr>
<td>Microscope Workstation</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
</tr>
<tr>
<td>e.g. Nikon Biostation</td>
<td>(Own Housing)</td>
<td></td>
<td>(Automation)</td>
<td></td>
</tr>
<tr>
<td>High Content Imager</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
</tr>
<tr>
<td>e.g. MD ImageXpress</td>
<td>(On Stage)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
<td>(<em><strong>) (</strong></em>) (***)</td>
</tr>
</tbody>
</table>

High level comparison of different imaging approaches for continuous live-cell analysis. *the relative scoring for ‘capacity, throughput’ and ‘automated analysis’ are made with reference to use for continuous live cell analysis rather than in end-point read mode.
assays to be miniaturized for low cell usage. In addition, the analysis packages provide have powerful functionality for fluorescence image quantification, although they are not especially configured for handling temporal data. Stand-alone, these types of systems do not have particularly good long-term temperature and humidity control (which is imperative for maintaining cell health) and the bright-field optics are limited. In an effort to increase throughput even further, several vendors have integrated robotic plate handlers to shuttle plates back and forth between the cell incubators and the microscopes/plate readers – this approach has had some success but is typically very expensive.

Somewhere between these two approaches lie medium to high-throughput solutions (live-cell imagers, microscope workstations) specifically targeted at the throughput/functionality/cost/ease-of-use sweet-spot for cell monitoring and kinetic assays. The key attributes offered by these systems are highly stable temperature and humidity control, the flexibility to work with a wide range of cell culture flasks and plates, fully automated image capture and analysis, and user-friendly software tools for the on-the-fly, real-time visualisation of cell behaviour.

**Live Cell Imaging & Automated Analysis Set Up**

IncuCyte® ZOOM Live Cell Imaging set up for continuous live cell analysis. The IncuCyte® system resides within a standard cell incubator for optimal environmental control, and captures images in situ from standard cell culture vessels (plates, flasks etc.). Images are automatically analysed in real time with user-trainable algorithms and visualised to report changes in biological parameters over time. Data show is a cell migration scratch wound assay – images are analysed over time for migration into the wounded zone and displayed as a full timecourse for each well in a micro-plate assay.
Delving Deeper Through Extended Cell Monitoring and Temporal Measurements

From the moment cells are placed in culture, whether freshly harvested or de-frosted from a cryo-vial, the learning opportunity begins. Typically, most laboratories do not tend to have formal processes for monitoring the cells prior to analysis; no measurements or images are captured and important questions such as “Are the cells supposed to look like that?” and “Have they grown or differentiated overnight?” are usually addressed subjectively, if at all.

Non-invasive, real-time monitoring provides a solution. It allows images to be captured from the instant the cells enter the incubator (and throughout the night), with the data used to track cell growth and morphology. The images can also be stored as a record for future inspection. When working with proliferating cells, real-time monitoring also allows researchers to track growth profiles and predict the optimal time for call passaging, while any unexpected changes in growth rate or morphology can also be observed. Some systems even allow you to do all of this from your desk or home, without disturbing your cells or even entering the laboratory.

When the time comes to prepare the cells for feeding or analysis, a number of unknowns can influence the outcome of the process. Real-time, non-invasive monitoring makes it possible to assess the impact of such experimental variables more accurately than ever before, so that they can be optimised for future studies. For example, by designing simple matrix experiments and monitoring the cells before and after changing a selected variable, optimisation becomes relatively straightforward and reliable. There is no need to set up individual time point measures or exhaust cells as part of the analysis process. The cells used in the matrix study are ready for use in downstream assays and optimal parameters can be re-used for subsequent experiments.

As an example, it is imperative to plate healthy cells evenly and at optimal densities across micro-assay plates for the assay to perform at its best. This is not always as straightforward as it may seem, particularly for miniaturized formats (e.g. 384-well plates) and/or when plating low cell numbers or co-cultures. In most cases, cells are plated for a period of time prior to the assay (often 24h) to give them time to recover before measurements are made. Monitoring the plates during this recovery time can provide significant insight and allows researchers to pinpoint the sources of within- and across-plate assay variance (e.g. uneven cell plating, poor cell health, washing artifacts etc.). Even within a small number of assay plates, it is usually possible to obtain a clear understanding of the relationship between assay signal and treatments, cell plating densities, plate coatings and other protocol parameters over time.

Capturing Rare Events
Where continuous monitoring really comes into its own is for capturing rare, stochastic, biological events – things that happen randomly and unpredictably. Considering stem cell workflows as an example, protocols for reprogramming adult cells into pluripotent stem cells typically involve introducing different
transcription factors (e.g. Yamanaka factors such as SOX4) and patiently waiting for stem cell colonies to form. Only a small fraction of the cells are successfully reprogrammed and colonies can emerge at any time during the process. It is lengthy and tiresome to inspect the plates every day for several weeks to find new colonies, while the constant moving of cells into and out of the incubator is detrimental to cell health. Using continuous monitoring techniques, cell cultures can be followed over time and observed remotely for emerging colonies. Taking this a step further, it is possible to use fluorescent gene expression reporters to pinpoint where and when relevant genes are switched on during the reprogramming and differentiation process. The ability to look at the morphological history of cells is particularly powerful when learning how to spot the best colonies and knowing when best to pick them. Similar principles can be applied to limiting dilution experiments for generating clonal cell lines (e.g. in antibody production) and cancer stem cell assays, where it is essential to know whether colonies originated from a single cell or not.

Whatever the cellular workflow, there is little or no downside to monitoring cells providing the overhead to the researcher is low. It is not essential to scrutinize every image that is captured. Only when something unexpected or untoward happens do you need to drill down into the image library to see where, when and what occurred. With real-time quantitative analysis of the images, the summary data can be trusted to highlight any changes in the cell parameters of interest.

**Workflow applications of continuous live-cell analysis**

- Cell Plating & QC
- Assay Development & Optimisation
- Phenotypic Assays & Screens
- Mechanism of Action Studies
- Gene Transfection
  - DNA, RNA
  - CRISPR/Cas
  - siRNA, miRNAs
- Labels & Reporters
- Reprogramming & Differentiation
- Clonal dilution

Interconnected cellular work flows supported by continuous live-cell analysis.
Comparison of typical cell workflows using traditional end-point (e.g. plate reader) and continuous live-cell analysis methods (e.g. IncuCyte ZOOM). In the continuous live-cell analysis paradigm, observations and measurements from cells are taken throughout the workflow to inform of changes in cell health & morphology, the impact of manipulations (e.g. transfection, plating steps) as well as the phenotypic parameters in the assay per se.

**Blurring the Boundaries of Cell Husbandry and Phenotypic Assays**

For researchers interested in higher order phenotypic cell measurement (e.g. apoptosis, cell migration, neurite outgrowth) the boundaries between preparing cells for the assay and the assay itself can be blurred. As an example, the time-course of neurite outgrowth from neurons in culture can be measured using non-invasive imaging methods prior to the addition of any test agents and used as a pre-treatment baseline against which the effects of treatments are measured. If the baseline parameters are not stable or appropriate for the assay for any reason (say, the neural network has not adequately formed yet), then treatments can be withheld or postponed until an appropriate time. In another case, if transfection of a reporter or marker such as GFP is required, the cells can be monitored throughout the transfection period prior to the assay and the timing of treatments can be judged to coincide with optimal reporter expression.

These workflows contrast with typical end-point imaging studies (e.g. high-content imaging ‘fix and stain’ protocols), where the parameter of interest is only measured at an arbitrary time point at the end of the experiment. Measuring changes non-invasively and over time in each well affords greater statistical precision than cross-well comparisons and allows researchers to design more effective drug association and washout experiments. Perhaps even more importantly, real-time monitoring means that researchers never miss the signal of interest, and can extract meaningful data about the kinetics of their biological system without additional cost or overhead.
**Example application of continuous live-cell analysis**

A549 cells were grown in culture and monitored for morphology and growth, prior to transfection with NucLight Red fluorescent protein. Stably transfected cells were then plated on 96-well round bottomed ULA plates at 5K cells per well to form 3D spheroids. The transfection, spheroid formation and growth process was monitored using IncuCyte live-cell analysis, prior to the assay on the effects of cytotoxic test drugs (e.g. staurosporine, SSP).

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**The Future of Cell Monitoring and Analysis**

To recap, in the search for more relevant and translational test systems, biomedical researchers are increasingly moving towards more advanced cellular models. These models display complex long-term dynamic biology, which places greater demands on the readouts and detection methods used to study them. Traditional end-point assays fall short and do not illuminate these parameter changes over time. In addition, the newer cell models have higher cost and ‘consumption’ considerations, which drive us to consider cells as ‘precious’ rather than throwaway. Together, these factors are creating a need for cell monitoring solutions that enable full temporal and long-term biological measurements, while minimising cell usage. Exciting developments in non-invasive detection technologies, particularly live-cell imaging, are beginning to address these needs and blur the lines between cell culture, cell manipulation and cell-based assay workflows.

So, what of the future?

Given its upsides, it seems likely that continuous live-cell monitoring and analysis will become the new gold-standard for cellular research, especially when compared to more traditional, end-point measures. By analogy with other research fields such as deep sequencing, the challenge will then rapidly shift to extracting knowledge from the data, rather than data gathering per se. ‘Big and Smart Data’ tools will be required to detect anomalies or trends that may otherwise be lost in the potential information overload. The hope and expectation is that the added insight provided by continuous monitoring will synergise with the extra sophistication of advanced cell culture and analysis systems to drive our understanding of cell biology and translational science to the next level.