Incucyte® Live-Cell Imaging and Analysis Best Practices

Technical Note

This technical note covers the following suggestions based on best practices for:

- Cell Seeding
- Well Volume
- Media Formulation and Exchanges
- Objects Within Light Path
- Empty Wells
- Vessel Positioning

Overview

The Incucyte® Live-Cell Analysis System is an automated image acquisition and analysis instrument that operates, captures, and analyzes images of cells from within a standard tissue incubator. Cells used in kinetic multi-well assays in the Incucyte® Live-Cell Analysis System typically run for extended time periods. As a result, cells must be maintained in a healthy state, with enough nutrients for the duration of the experiment, as well as proper assay set-up to ensure conditions for high quality, reproducible image acquisition of cells over time. These factors, which make for best assay practices when studying cell biology in a long-term kinetic fashion, are not necessarily required for traditional end-point/single time-point analysis. However, all parameters should be optimized in order to achieve superior assay performance.

Best Practices – Cell Seeding

Cell Density: Frequently dictated by the functional assay being performed, cell density is a key player in assay performance. As cells approach 100% confluence, contact inhibition will begin to slow growth and can impact cell health. For most assays, a low density range for both adherent (1,000-5,000 cells) and suspension cells (5,000-30,000 cells) should be considered during assay optimization. Some assays are density driven, such as Incucyte® Scratch Wound Migration and Invasion Assays, and require confluence to be closer to 100%. In general, it is crucial to optimize the cell density for each assay type as this will ensure high quality images can be acquired, processed, and analyzed to achieve reproducible, robust data.

Cell Settling: Uneven cell distribution can have a significant impact on the quality of data obtained in a live-cell assay due to the potential for variance both in and between wells. Cells accumulating at well edges or concentrating in the center of a well are commonly observed phenomena, in particular in outer wells, leading to some researchers to not use these wells due to concerns about data reliability. Cell distribution within a well is strongly determined by convection currents which circulate within a well during warming of culture medium. If cells are present in suspension at this moment of fluid flow, they are more likely to be moved by these currents. To eliminate or reduce the impact of this effect, it is strongly recommended to allow cells to settle on a flat surface at ambient temperature following cell seeding for at least 20 minutes (45-60 for suspension cell types in assays such as chemotaxis). This settling period allows cells to sediment to the base of the well and interact with tissue culture plastic or surface coating, limiting the movement of cells and creating a more homogeneous cell distribution.

Surface Coating: Surface coatings have increased importance for long term assays because it can impact the growth and health of cultures over time. If these conditions are not properly optimized, it can impact the performance of the assay. For some functional assays, like proliferation studies, non-adherent (suspension) cell lines should be adhered to the vessel surface using a coating like poly-L-ornithine (PLO). Adherent cell lines typically do not require a coating. In more complex functional assays, like neuronal assays, stem cell assays and chemotaxis studies, a surface coating material might be required for both adherent and non-adherent cells alike. Some examples of coating material include poly-D-lysine, poly-L-orthinine, laminin, fibronectin, or gelatin. Our assay specific protocols provide more details around surface coating optimization suggestions per 2D and 3D applications.

Best Practices – Well Volume Plate Type

The volume of liquid within a well is critical to consider, not only for maintaining cell health, but also for eliminating deformation in image acquisition. Too low of a well volume will cause poor image quality, even with Incucyte's ® algorithm to correct for deformation in transmitted light. In general, all assays will benefit from using more volume in the wells, when applicable. The Incucyte® Live-Cell Analysis System is able to support over several hundred vessel types based on the application or software module; however, we have highlighted below commonly used microplates and their recommendations for assay set up and image acquisition.

Plate Type

- **96-well plates:** We typically recommend a range of 50-200 µL medium per well. The lower end of that range can be used for short-term assays (e.g. 24 hours) while the higher end should be used for longer assays or when media exchanges are required. The standard well volume we use in house is 100 µL.

- **384-well plates:** We typically recommend a range of 40-80 µL medium per well. The lower end of that range can be used for short-term assays (e.g. 24 hours) while the higher end should be used for long-term assays (> 48 hours) or when media exchanges are required. A standard well volume we use in house is 60 µL.

- **Flat vs. round bottom:** Round bottom plates can hold more medium than flat bottom plates and are typically used in Incucyte® Single Spheroid Assays. Note that for long-term assays, more volume will also aid with partial media exchanges.

Assay Set Up

- **Assay Type:** For some applications, shorter assays can benefit from lower volumes per well so long as users do not go too low, where image quality will be impacted. For some of our functional assays, like spheroid and chemotaxis, volume amounts will be noted in the protocol for best assay practices. We typically do not recommend using lower than 40-50 µL of medium.

- **Assay Duration:** Users should take into consideration the length of the assay to support cell health. Longer assays or assay where users will have to perform media exchanges should have a higher volume of media present during experiments.

- **Scan Frequency**: Scheduling scans at a higher frequency can lead to a greater amount of heat generation due

to the mechanics of the Incucyte® system. This may in turn increase chances of evaporation. Therefore, with a very active scan schedule, more volume in the wells is recommended.

Best Practices – Assay Media

Media Formulation: In traditional end-point assays, researchers typically optimize a functional assay with their preferred cell lines and preferred cell line specific medium. That said, there are cases where users might want to transition cells to an alternative media for live-cell imaging. For example, riboflavin can cause high levels of background fluorescence when imaging in the green channel. Some assays would be better served by using a low-riboflavin media in place of media with a higher riboflavin content for imaging reasons. Additionally, cell line properties may change when grown in different media formulations, and these changes can be critical when running longer term assays; for example, cells may proliferate and/or migrate differently in different types of media. For this reason, it is recommended to optimize media formulations for the best cell line performances, in particular for assays such as chemotaxis. When preparing medium, it is recommended to filter additional components, e.g. FCS, with a 0.22 µm filter (such as the Sartorius Minisart®).

Media Exchanges: Re-feeding cells is a typical workflow in cell culture maintenance, where cell medium is exchanged every 2-3 days. This practice should be continued in long-term kinetic assays that go beyond 72 hours to maintain essential nutrients for cell health. However, there are assays were re-feeds will not be possible, such as toxicity assays and phagocytosis bioparticle assays. Assays where the cells begin to lose membrane integrity will also not be served well by a media change. Finally, assays using suspension cells, even when attached to a surface, will also be affected by media exchanges. It is important to understand the nature of your assay and its duration to account for any media changes that may be required. For some sensitive cells, such as neurons, we recommend doing a half media change rather than a full to preserve the samples.

Best Practices – Objects Within Light Path

As the Incucyte® Live-Cell Analysis System is an imaging system utilizing transmitted light, any object within the light path can interfere with it and generate artifacts within the images. As such, we have a number of recommendations to prevent these artifacts occurring:

Bubbles: Bubbles are high contrast objects which can interfere with the light path of an imaging system and impact data analysis in the Incucyte®. We recommend the use of reverse pipetting to limit bubble formation when preparing vessels for imaging in the Incucyte®. Should bubbles still be present, we suggest removing the inner straw from a standard laboratory wash bottle, adding a little 70% ethanol to the bottle, and blowing the vapor over the wells to break the bubbles. The ethanol is sterile so will not contaminate a sample and will break the surface tension of the bubbles.

Condensation: A standard effect of transferring a tissue culture vessel from the culture hood to a warm, humid incubator is condensation on the plastic of the vessel. This can lead to series of water droplets which can impact the transmission of light through the vessel. To prevent this from occurring, we recommend allowing the vessel to warm for at least 20 minutes to allow condensation to evaporate before imaging commences. An alternative solution to allow immediate imaging is to pre-warm an additional vessel whilst preparing the sample vessel. The lids are then switched so that the warmed lid is used in the Incucyte® assay allowing imaging to commence immediately.

Fingerprints, Markings, Scratches

- Vessels should be handled with gloved hands on the sides of the vessel, not on the top and the bottom due to the potential for residue to be left on the vessel.
- All labelling should be made in areas where imaging will not occur, for example, the sides of the plate.
- Vessels with a large number of scratches in the plastic should be avoided. Scratches are high contrast objects that can interfere with both focusing and masking. As such, we recommend that if batches of plates contain a large number of scratches, then they should not be used for live-cell imaging.
- We suggest any media droplets on the lid of the vessel are carefully wiped away with a lint-free cloth.

Best Practices – Empty Wells

The Incucyte® Live-Cell Analysis System utilizes an image based autofocusing approach to determine the most appropriate z-plane in which to obtain an image by assessing contrast levels throughout the z-axis. To optimize this process, information from preceding wells is used to inform the system as to the most likely focal plane in which to image in subsequent wells. If however there are no cells present within the well, there is the possibility that the Incucyte® will struggle to find a focal plane or find contrast in a focal plane away from that of the cells in downstream wells leading to an out-of-focus image. To avoid this possibility, and to speed up scan times, users can simply select a scan pattern which corresponds only to the wells which contain cells when setting up a vessel in the Add Vessel guided interface within the software.

Best Practices – Vessel Positioning

When loading vessels into the Incucyte® Live-Cell Analysis System, we recommend removing the metal tray which holds the vessel from the Incucyte® gantry and placing it on a flat surface. This allows correct positioning of vessels in all trays and downwards pressure to be applied when using multi-well plates, with well A1 positioned in the top left corner. This is important for fixing a vessel in position due to the ball bearings in this tray type which hold the vessel in location. Inserting a vessel whilst in situ can lead to vessels not being seated correctly within the tray. An exception to this is the situation where a tray is already occupied with an existing vessel. In this circumstance we would recommend giving resistance to the underside of the tray, preventing downward pressure being applied to the gantry arm and allowing the vessel to be seated correctly without impacting an existing experiment.

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