Application Note

Clone Imaging and Proof of Monoclonality

Solentim

Introduction

The verification that a new manufacturing cell line developed to produce a protein therapeutic is derived from a single cell (i.e. is clonal) is an absolute requirement to ensure product consistency and to achieve optimal yield in downstream bioreactors. Historically, using statistical methods for dilution and colony outgrowth was sufficient, but nowadays drug regulators and clients for upstream cell line development groups alike want to see documentary evidence of a single cell.

Many cell line development laboratories have replaced manual methods involving staff looking down microscopes to identify single cells with automated imaging systems in an attempt to increase throughput. However, the images generated have not always been of sufficient quality to confidently discriminate a single cell from debris and other artefacts in the microplate.

This has resulted in a proportion of potentially high producing clones being discarded or having to go through a lengthy round of subcloning to establish monoclonality.

As such, previous generation imagers are satisfactory when looking at colony outgrowths and measuring confluence, but unsuitable for consistently identifying wells which contain a single cell.

This application note illustrates how the Cell Metric™ imager can identify single cells before division on the day of seeding (Day 1) (**see Figure 1A below**), while also providing a clear image of the cell and its subsequent growth for documentation.

Materials and Methods

The platform used for this application is the Cell Metric imager in three possible formats: either as a stand alone unit, or as part of an integrated robotic work cell with a robotic arm and incubator, or the Cell Metric CLD which has a built-in incubated stacker for batches of 10 plates and enables walk-away operation for 40 minutes.

Cells are the typical suspension cells employed in CLD groups, such as CHO-S, or CHO DG44, which are seeded by

Figure 1: Whole well image of 96 well plate with inset zoom images of the cells:
A) Day of seeding – single cell; **B)** After 24 hours – pair of cells following cell division.

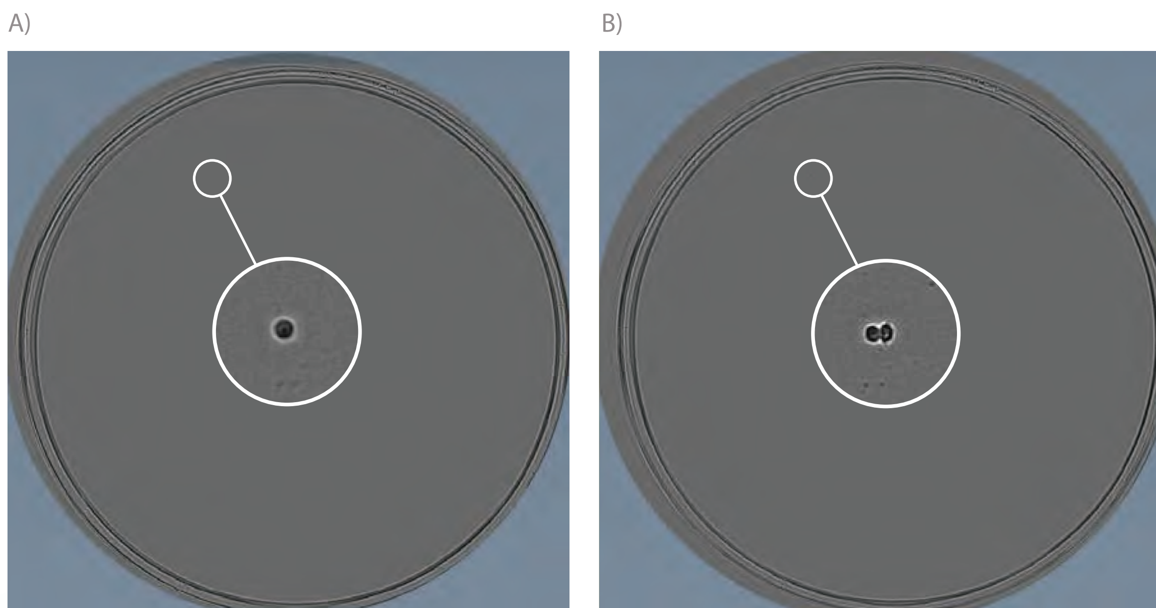

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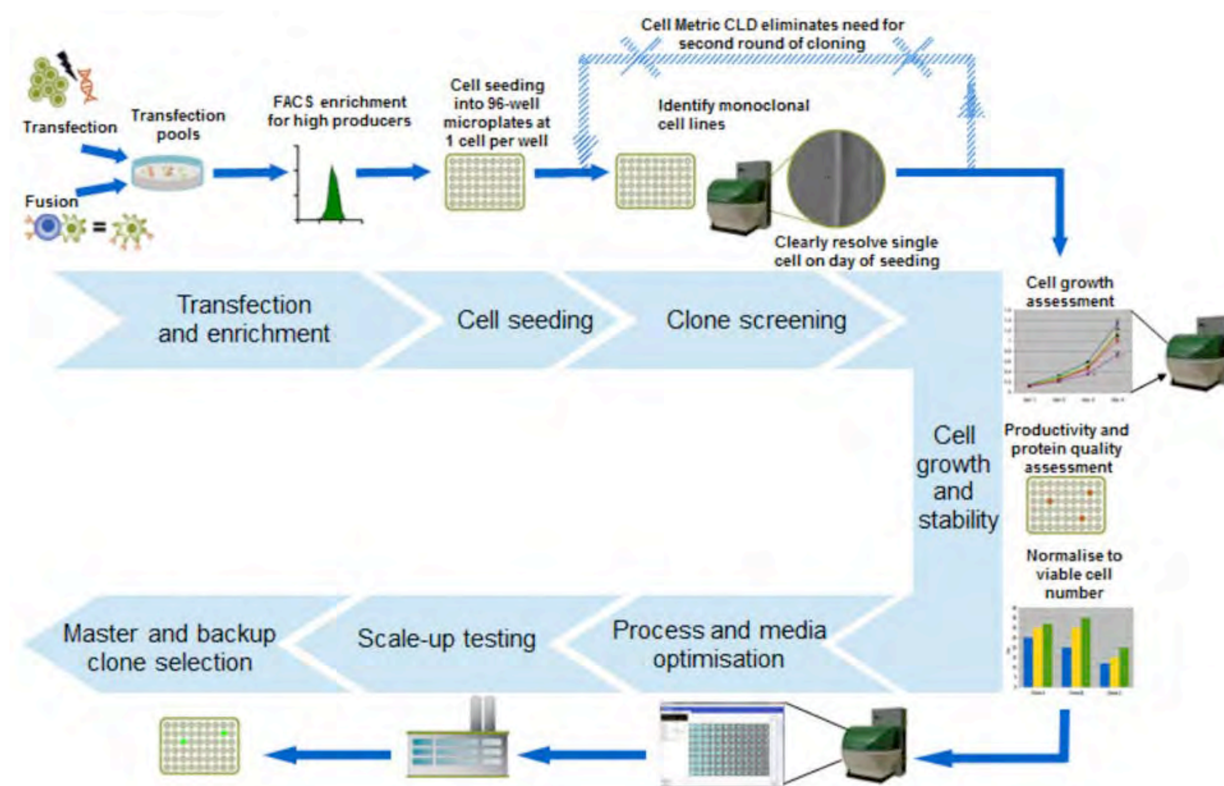
Figure 2: Workflow when clones are seeded by FACS or limiting dilution.



limiting dilutions (LD) or flow cytometry (FACS) e.g. using a BD FACS Aria (Becton Dickinson), aiming for a single cell in each well of either a 96 well plate or, in some instances, 384 well plates (**see Figure 2**).

Choice of plate is important for the best imaging results. Typically a flat-bottomed tissue culture plate or occasionally a U-bottom plate can be used. Plate quality and handling are important to minimise the contamination of plates by foreign objects and to avoid finger prints on the base of the wells. It is also useful to filter the tissue culture medium to remove particulates which could otherwise show up in the images. Wells should typically be around two-thirds full with media to avoid the meniscus at the top of the well causing shadows when imaging.

After seeding, cells need to settle to the bottom of the well to be ready for imaging, as the Cell Metric will focus on a monolayer at the base of the well. Cells can be allowed to settle passively which takes at least 2 hours, or the plates can be centrifuged at 200 RCF for 2 to 5 minutes to encourage the cells down to the well bottom.

The Cell Metric software organises all of the settings that are required to scan a batch of plates into a'scan profile'. A profile can be set up in advance and reused for each plate in a batch. The scan settings include parameters to specify the microplate type being used, the focus offset to the bottom of the wells and the illumination settings for optimal image quality. For monitoring colony growth, cell detection parameters can also be adjusted. Once a scan profile is set up in the software, it can be selected from the menu, with the user pressing a single button to scan the plate. Plate identification is achieved either by reading a barcode on the plate or from being manually typed in by the user. The plate ID is used to update the history of the plate so that well growth curves and time course images for each well, from cell through to colony, can be easily generated.

Images are captured on the day of seeding (Day 1) as soon as the cells have settled. This is the essential time point, which is later used to establish clonality before the cells have had a chance to divide. It is not important if cells subsequently move later on when plates are handled, so long as the first image shows a single cell. Images can then be captured on subsequent days to monitor the presence and rate of colony growth. Confluence data can be used to quantify colony growth and to determine which wells contain colonies whose supernatants will be assayed for protein secretion or productivity, using an ELISA assay, an HTRF assay or the ForteBio Octet (Pall Corporation).

The Cell Metric uses two different imaging modes for different time points during the workflow. On the day of seeding the "verify clonality" mode is used, which results in a higher

contrast and takes approximately four minutes per 96 well plate (depending on plate type). For subsequent days, the "clone growth" mode is used and gives a faster speed per plate of around 3 minutes.

A typical cell line development project will run over several months and generates a large amount of image data. Hence it is important to practically consider how this data will be stored and managed from an IT perspective. Each whole plate image in high resolution mode is around 150 MB. A batch of 50 microplates, each imaged at four time points will therefore generate around 30 GB of data. This is easily handled by the Terabyte hard drive of the Cell Metric computer, while it can also be stored cost effectively on an external back-up hard drive. If required, the Cell Metric can store the scan results directly onto a network drive, thus removing the need to copy the image data off of the Cell Metric PC at the end of a project.

Data and Discussion

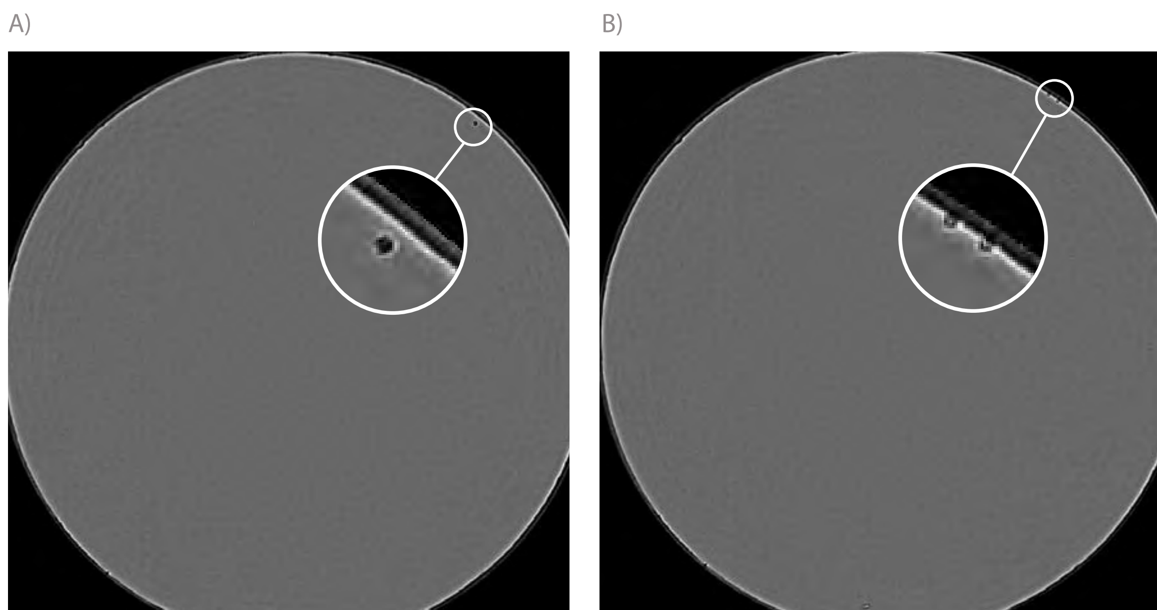
The key to this process is to generate and store an undisputed image of a single cell on the day of seeding. The Cell Metric is able to achieve this using bright field imaging only, with no requirements for fluorescence or other labels. There are several key aspects of the imager which make this application possible:

1. High resolution and high contrast imaging to observe a single cell (**see Figures 1 and 3**).
2. Accurate focussing and contrast - cells clearly show up against the media background (**see Figures 1 and 3**). Using proprietary technology, the Cell Metric tracks the contours of the base of the microplate to correct for any distortion of the plate plastic that may have occurred during manufacture, so that all images across a well and all wells across the plate are always completely in focus.
3. Even illumination right up to the edges of the wells, where the cells often accumulate. (Note the clear well edges in Figures 1 and 3).
4. Multiple images are captured per well and seamlessly stitched together to form a single continuous, high-resolution image.

Examples are shown of single cells imaged on the day of seeding, which have subsequently divided into two cells 24 hours later for both 96 well plates and 384 well plates (**see Figures 1 and 3**).

Different customer workflows will use the image captured on the day of seeding in different ways. Some customers will track images every day after seeding for colony outgrowth and then review the Day 1 image to establish clonality. This may facilitate the earlier selection of clones to take forwards. Other customers may do little intervening imaging between Day 1 and say Day 14, choosing to advance all colonies first to assess growth rate and amount of protein secretion, before then going back and checking the Day 1 seeding images of only the subset of best producers to establish clonality.

Figure 3: Whole well image of a 384 well plate with inset zoom images of the cells on the edge of the well:
A) Day of seeding – single cell; **B)** After 24 hours – pair of cells.



Conclusions

The image of a single cell provided from the Cell Metric provides the traceability and documentary evidence that a cell line developed for biotherapeutics or biosimilars is clonal. Within an organisation, it also provides an audit trail which gives confidence for downstream production groups.

The other major benefit is an economic one, as an image clearly showing a single cell eliminates the need for a second round of sub-cloning. This can significantly shorten the cell line development process, in some cases by several months per project. Customers can see gains in productivity as a result, as more cell line development projects can be achieved each year for a fixed head count. This provides a very clear return on investment and solid justification for implementation of the Cell Metric.

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