Methods for fluorescence qualification of your cloning methods

Solentim
Clarity in Cell Line Development

Introduction

Image verification of a new manufacturing cell line developed to produce a biologic derived from a single cell is an absolute requirement for Chemistry, Manufacturing and Controls (CMC) in order to ensure product consistency prior to manufacturing and to achieve optimal yield in downstream bioreactors.

Laboratory method validation and verification to provide proof that the entire process being implemented is robust, repeatable and provides proof of clonality are of high importance to users, CMC Directors and Regulators alike.

The Solentim Cell Metric® is currently widely used for the image verification of clonally-derived cell lines using its bright field imaging capabilities. Given the high quality of bright field imaging, the Cell Metric has not required the use of fluorescence staining to identify single cells. Indeed, the use of fluorescence labelled cell lines for the production of biotherapeutics would not be advocated by the regulatory authorities.

The latest Cell Metric platform has now been enhanced with fluorescence specifically for the applications involved in qualification and documentation of cloning methods in cell line development.

Methods

The Cell Metric's fluorescence enhancements have been designed with flexibility in mind enabling the user to apply additional quality control and/or validation of their chosen seeding method with the use of single or dual fluorescence cell labelling.

The Cell Metric has been specifically optimised for use with CellTracker Green[2] and CellTracker Red[1] cell dyes. Other dyes or fluorescent expression systems such as Green Fluorescent Protein (GFP) or Red Fluorescent Protein (RFP) can also be used.

Imaging using the Cell Metric:

Fluorescence on the Cell Metric is uniquely designed for detection of 'rare events'. As with standard bright field imaging to verify clonality, a scan profile specifically using fluorescence (Green and Red) can be set up to include all the required parameters to image the microplates using bright field and fluorescence. Whether imaging wells containing a single colour population or a mixed colour population, the images will be captured first in bright field mode, and then using the fluorescence channels.

Microplates can also be imaged at a single time point or multiple time points depending on the user's requirements and the fluorescence labels.

In this experiment and in most cases when using a CellTracker stain, the stain should dissipate from the cells within a 3-6 generations, so it is ideal for use in the validation/quality control of the chosen seeding method. In combination with the imager, the stain will essentially aid the rapid cell detection at Day 0 imaging. The microplate can still be
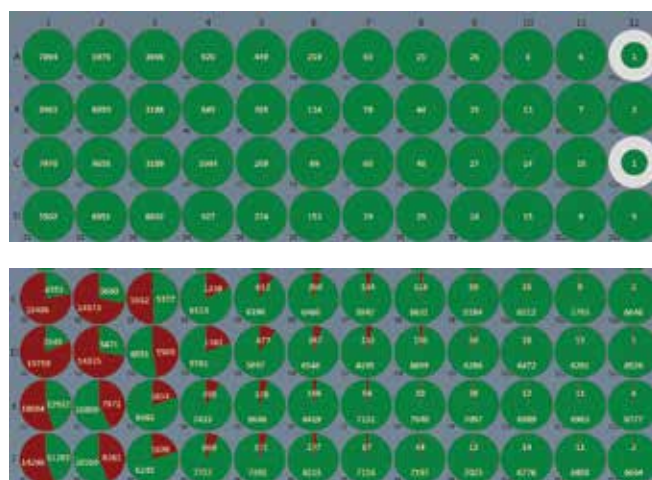

Figure 1: Representation of wells containing multiple fluorescing cells. Top) Single channel output. Bottom) Dual channel output.

repeatedly imaged over time, without the fluorescence, to provide the standard time series of the cells growing into a colony, which can be provided as evidence of a viable clonally-derived cell line using the Cell Metric Clonality Report.

If there is a requirement to continue imaging where fluorescence will continue to be visualised/expressed from the cells (for example; for those users with GFP or RFP expression vectors), the system also has the capability to continue imaging using the fluorescence channels and display the number of loci per well in the chosen fluorescence channel (see **Figure 1**).

Example protocol for staining and plating the cells:

CHO cells labelled with CellTracker Green and CellTracker Red were used.

1. 10mM solutions of CellTracker Green and CellTracker Red were prepared as per the manufacturers instructions[1,2].

2. Working solutions were then prepared by diluting the labels to the appropriate concentration in the CHO culture media.

3. CHO cells were counted and the appropriate level of cells were aliquoted into two separate tubes.

4. CHO cells were spun down, the media removed and the cells re-suspended in CellTracker Green and Red working solutions.

5. The two cell preparations were incubated at 37°C for 30 minutes.

6. The CHO cells were then spun down again for removal of the CellTracker working solutions, and finally re-suspended in the CHO culture media.

7. The two cell populations (now stained with CellTracker Green and CellTracker Red) were either pooled or kept separate for the appropriate experiment.

8. The appropriate level of the cell suspension was then added to 96 and 384 well microplates to provide single cells per well and allowed to settle before being imaged on the Solentim Cell Metric.

Cloning method validation using two colour fluorescence staining:

Using the routine cloning method of choice e.g. FACS dispensing or Limiting Dilutions, the mixed cell population can be plated across several microplates and imaged at Day 0 using the new fluorescence features to detect rare events. Once captured, the images can be displayed for review in bright field only, in fluorescence only and in composite mode (combined bright field/fluorescence overlay).

Figure 2 displays a whole well image from a 384 well microplate well containing a CellTracker Green-stained CHO cell and a CellTracker Red-stained CHO cell. Inset is shown the detection of cell candidates in each of the imaging channels; bright field, red and green. Cell candidate detection is carried out in a similar manner to that of standard bright field only cell candidate detection, however, the fluorescence signal is taken into account within the algorithm to further distinguish fluorescing objects.
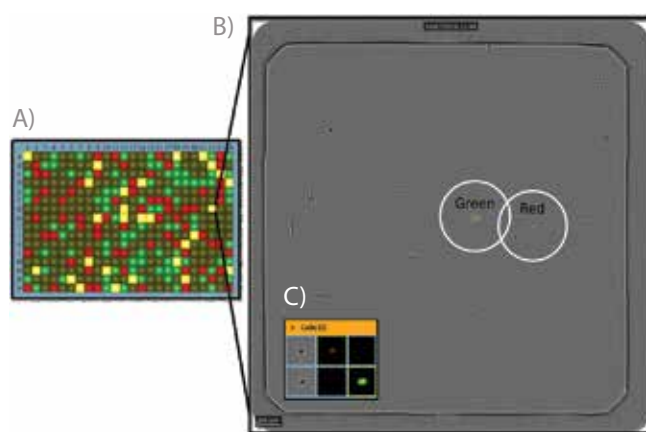

Figure 2: Representation of the software output of the dual fluorescence cell detection capabilities of the Cell Metric for cloning method qualification. A) Whole microplate representation of the fluorescent objects detected per well. B) 384 whole well composite image of bright field, green and red fluorescent images. C) Cell candidate representation for all channels used; Left = bright field image thumbnail, Middle = red image thumbnail, Right = green image thumbnail. Top line of thumbnails = cell candidate 1. Bottom line of thumbnails = cell candidate 2.

For every microplate imaged, an overview of the result is displayed as a microplate map, highlighting where fluorescent objects have been detected. This overview will enable a quick visual determination of the cloning method results: the number of empty wells, wells containing a single fluorescing object, wells containing more than one fluorescing object and also more than one cell colour. These statistics for each microplate are presented in a microplate statistical summary for a quick assessment, of the seeding method output (see **Figure 3**).
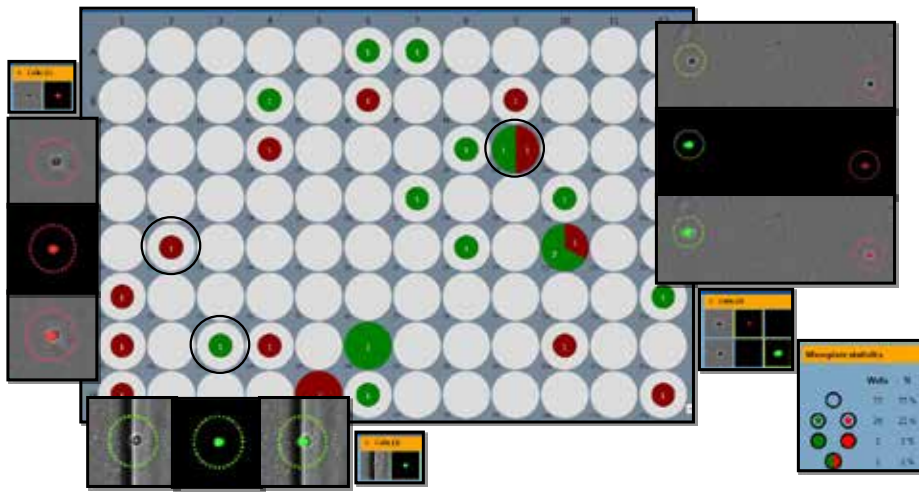
Figure 3: Representation of the images produced for review on Day 0 (time of seeding) when imaging a 96 well microplate using bright field imaging combined with dual fluorescence (green and red) imaging.

Statistical probability analysis can be drawn from this data by identifying those wells that contained two separate and distinct cells plated, versus identifying those wells containing two cells that originated from a single cell that was in the process of dividing when deposited.

To continue on from this, if using GFP/RFP vector expression systems to label the populations, multiple time points can be imaged (using fluorescence) and the prevalence of "ghost wells" can be determined at a later date. Ghost wells (the appearance of a clone growing in a well that didn't contain a cell on day 0) are caused by the possibility of cells either stuck to the well wall or still in transit to the bottom of the well which is outside of the focal plane for image verification. For example, a comparison can be made to determine if the colours seen in the well images at the end of the experiment,

are the same as the colours seen at the beginning of the experiment. If a well starting with green-only objects ends with a mixed green-red population, it can be determined that this was a ghost well and calculations can be made to determine the overall incidence of seeing ghost wells in the seeding method.

Not only can these fluorescence detection methods be used for validation the cloning method, they can also be used on a regular basis to QC the seeding method to ensure it is still performing as expected.

Implementation of routine quality control of seeding method:

Throughout daily/weekly/project based use of the system, regular quality control checks of the seeding method
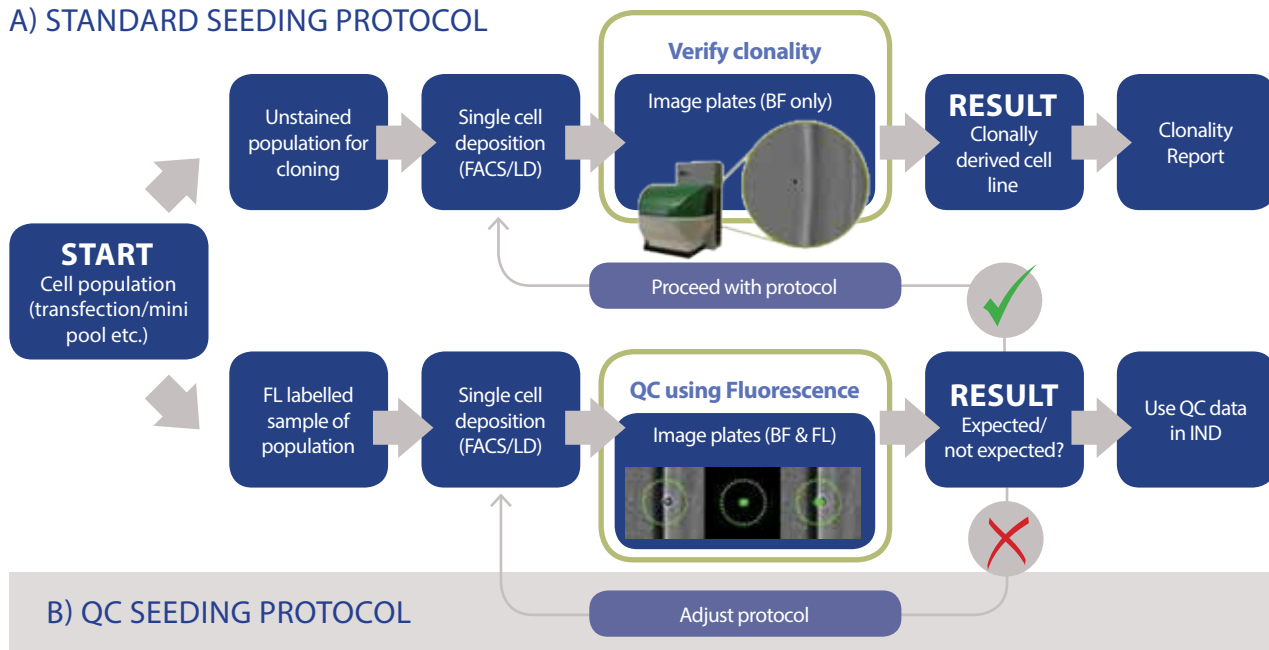

Figure 4: Workflow overview outlining how regular quality checks can be used to ensure the seeding protocol is performing as validated/expected. The process starts with the heterogeneous population which can be fluorescently stained (workflow B) separately, either before or during standard seeding protocol (workflow A), to check that the protocol is producing the expected deposition.

can be carried out using the Cell Metric's Fluorescence detection parameters. When depositing single cells using FACS, Limiting Dilution or other methods, there is often a pre-determined expected rate of single cell deposition (as defined by your method validation – see previous section). The process map in **Figure 4** outlines a way in which the seeding protocol can be QC checked to ensure the method is still consistently producing the expected results in line with the method validation results.

Either before or during a single cell deposition round, an aliquot of the same cell population (fresh transfection/bulk pool/mini pool) can be stained with a single fluorescent label (CellTracker) and deposited as per the standard seeding protocol. This labelled sample can then be imaged using the fluorescence application, and the results assessed to ensure that the rate of single cell deposition matches what is expected (see **Figure 5**). If using Limiting Dilution this assessment could be in line with the Poisson distribution or if using FACS this could be in line with value obtained when the cell deposition settings were originally optimised.

Figure 5:
Representation of single colour seeding method output for quick review.
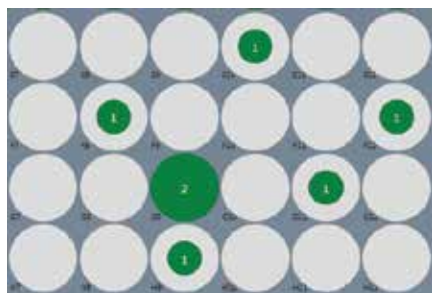
If the results were not as expected, for example in the case of FACS there were more than expected doublets, or in the case of Limiting Dilution there were more than expected empty wells, an adjustment could be made to the settings or seeding protocol respectively to ensure the expected results are achieved.

If the protocol is well established, the QC check could be carried out in parallel to or as part of the standard unstained cell seeding experiments to confirm the deposition rates for further supportive data in the IND submission.

Discussion and Conclusions

There are various cloning methods used in cell line development and they each have their own inherent drawbacks in terms of seeding efficiency and cloning efficiency. The high quality of bright field imaging on Cell Metric has consistently proved that single cells can be verified accurately, including around the well edges, without need for fluorescence. These bright field only results can, to a degree, already be tallied to theoretical single cell distribution across the microplate e.g. a Poisson distribution. The need to avoid fluorescence has also been desirable as some previous reports have indicated that CellTracker labels may inhibit colony growth or even be cytotoxic. Finally, given that most biologics will be injected into patients, extraneous fluorescence labels are also deemed undesirable from this aspect as well.

Presented here is a suggested method for an improved approach to qualifying the cloning method using dedicated fluorescence applications.

For routine seeding and image verification, we propose that all this can and should still be performed in bright field only mode on the Cell Metric, given that the cloning method will be fully qualified in advance and will have these supporting numbers/statistics as part of the IND package.

References

1. Thermo Fisher Scientific CellTracker™ Red CMTPX, Catalogue Number: C34552.

2. Thermo Fisher Scientific CellTracker™ Green CMFDA, Catalogue Number: C7025.

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