Technical Overview

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Limiting Dilution Method Using the Agilent xCELLigence RTCA eSight System

Authors

Tian Wang, Grace Yang, and Peifang Ye
Agilent Biosciences Co. Ltd.
Hangzhou, China

Rashmi Pillai, Ryan Raver, Alejandra Felix, Nikhil Mittal, Nancy Li
Agilent Technologies, Inc.
San Diego, CA, USA

Abstract

Monoclonal stable cell lines are widely used in the biopharmaceutical industry to produce recombinant proteins and monoclonal antibodies, as well as to perform drug screens. Drug regulatory agencies require demonstration of stable cell line monoclonality, as it is critical to guarantee product quality. There are many available methods to create monoclonal cell lines, each with a balance of benefits and deficiencies. This technical overview describes a method for monoclonal cell isolation using a live cell analysis system, the Agilent xCELLigence RTCA eSight, with a limiting dilution cloning method. Automated real-time imaging with the xCELLigence RTCA eSight enables validation of the monoclonality of cell lines with minimal hands-on time.

Introduction

Single-cell cloning is used to isolate individual cells from heterogenous populations based on their characteristics. Both the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the United States Food and Drug Administration (FDA), state that recombinant products must be cloned from a single cell progenitor.[1,2] At present, common methods to obtain monoclonal cells include flow cytometry-based cell sorting and the limiting dilution/dilution cloning method.[3] While flow cytometry cell sorting enables cell screening by detecting and measuring individual cell size, granularity, and fluorescence, multiple rounds of flow sorting are often required to obtain single cells and can compromise cell viability and yield.[4,5]

Another relatively simple, convenient, low-cost, and cell non-perturbing method to obtain single cell clones is limiting dilution. Without imaging equipment, at least two rounds of limiting dilution (dilution ratio 0.3 to 0.8 cells per well) are required to obtain high monoclonal rates.[4] Manual screening of wells is also recommended to rule out the presence of cell clumps, an additional labor-intensive step. Incorporating the use of an automated imaging system while limiting dilution improves the success rate of obtaining single cells, increases screening speed, and reduces reliance on manual analysis.[3] The xCELLigence RTCA eSight Dilution Cloning Module enables users to rapidly identify and document cell line monoclonality.

Assay principle

The xCELLigence RTCA eSight is currently the only instrument on the market capable of performing live cell imaging and highly sensitive cellular impedance measurements to monitor cell health and behavior in real time. The instrument includes five cradles. The first three (cradles 1 to 3) use specialized gold biosensor plates (E-Plates) to collect impedance and imaging data simultaneously. The other two cradles (4 and 5) are suitable for typical cell culture plates and only collect imaging data. Cellular impedance is recorded at a user-defined time frequency (every 15 minutes by default) and reported using a unitless parameter called Cell Index.

This technical overview describes a single-cell screening method in common 96-well plates using the RTCA eSight system. All assays were performed in cradles 4 or 5. A new assay type, *Dilution Cloning*, was developed, wherein the whole well is imaged using a 5x objective, providing two focusing modes: brightfield-based and fluorescence-based (red or green). After choosing imaging channels, users can select the appropriate focusing mode, depending on the presence of fluorescent labels in screened cells. After selection, imaging is collected according to specified channels. Fluorescent focusing is preferred as it yields higher quality images. After single-cell deposition, whole well images are captured automatically for 10 to 14 days, while eSight software image processing recognizes cells with simple training. Cell growth plots combined with images are then used to identify wells containing monoclonal cells.

Materials and methods

HT1080 Red cells were produced by transducing parental cells with Agilent eLenti Red (part number 8711011) to express nuclear-localized red fluorescent protein (RFP). After 72 hours, the medium was changed to complete growth medium containing 2 µg/mL puromycin for 14 days to select for transductants.

HT1080 cell maintenance and assays were conducted at 37 °C and 5% CO_2 in EMEM (ATCC, part number 30-2003) with 10% FBS (Gibco, part number 10099-141) and Penicillin/Streptomycin (Hyclone, part number SV30010).

Limiting dilution cloning

HT1080 Red cells in the logarithmic growth phase were collected and a cell suspension of 1×10^5 cells/mL was prepared. Through consecutive 10-fold dilutions, the cell density was adjusted to 1×10^3 cells/mL. Then, 200 µL of the cell suspension was further diluted in 10 mL, to a cell density of 2 cells/100 µL. A 100 µL amount of the suspension was added to 96-well plates containing 100 µL of medium. After allowing cells to settle for 30 minutes at room temperature, plates were placed in cradle 4 or 5. The assay type was set to Dilution Cloning and whole-well images were captured every 4 to 6 hours with a 5x objective. Plates were imaged for 10 to 14 days to monitor clone formation.

Results and Discussion

Identification of monoclonal colonies using brightfield imaging

The brightfield focusing mode of the Dilution Cloning module can screen cells without fluorescent labels. The Plate View feature on the Image Browser tab enables individual well selection for detailed analysis. Wells with no cells or with multiple cell clusters were excluded (for example, wells E5 and G8 in Figure 1A), as the latter likely arose from multiple cells. For wells with single clusters, images at earlier time points were reviewed to assess clonality. As shown in Figure 1B, the cluster in well D1 grew from a single cell, while the cluster in well G2 (Figure 1C) was revealed to not be monoclonal, as four cells were originally deposited on Day 0.

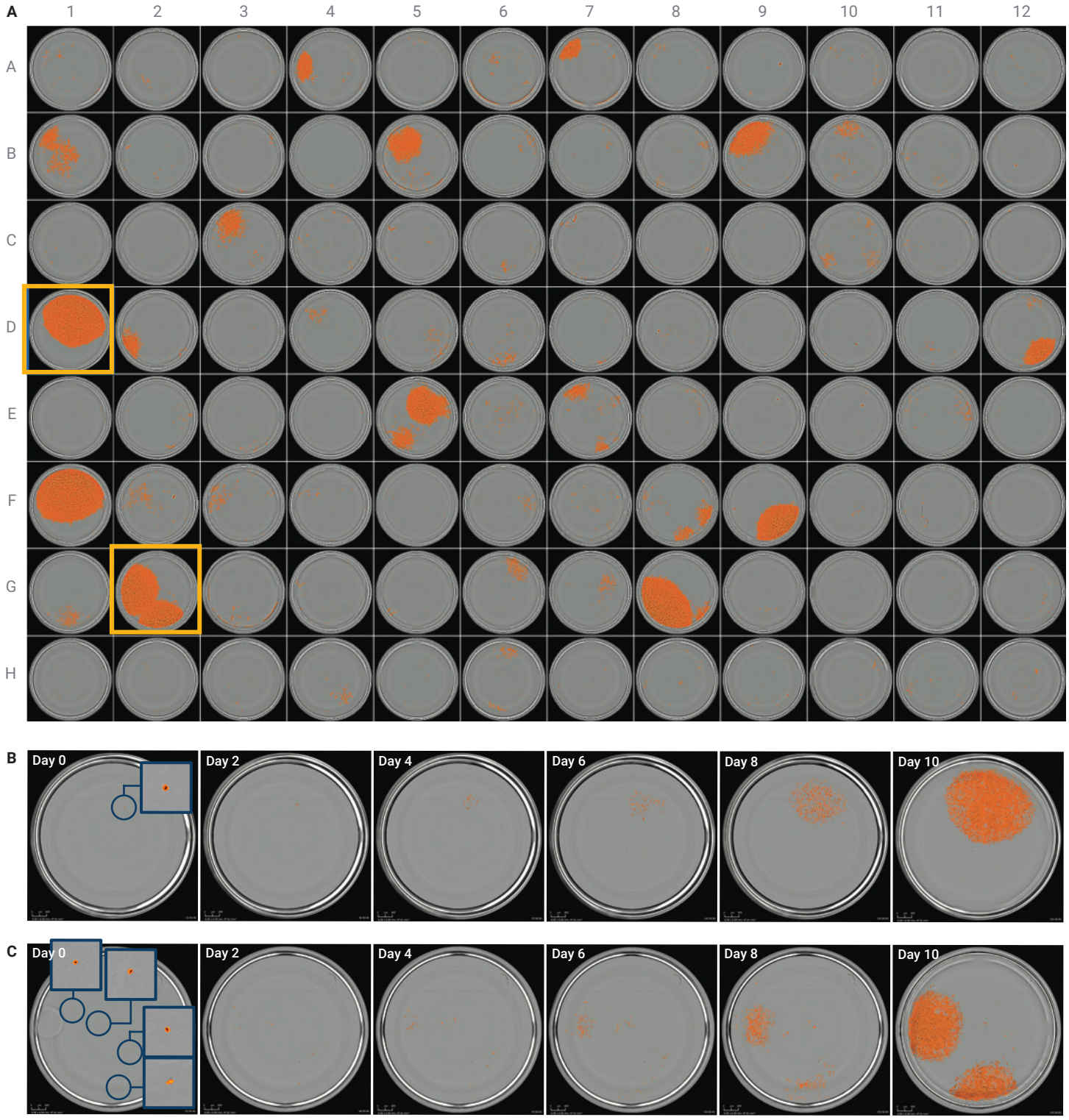

Figure 1. Screening of monoclonal cell lines based on data collected by eSight. (A) Plate view of cell clusters 11 days post cell deposition (brightfield recognition mask in orange). (B) Images at various time points from well D1 demonstrate cluster is monoclonal. Blue box in the Day 0 panel shows an enlarged image of a single cell. (C) Images at various time points from well G2 demonstrate the cluster is likely not monoclonal. Blue boxes in Day 0 show enlarged images of four single cells.

Figures 1B and 1C demonstrate the ease with which single cells are captured by the Dilution Cloning Module.

Identification of monoclonal colonies using fluorescence imaging

As it collects brightfield images, xCELLigence RTCA eSight can simultaneously collect fluorescent images. Figure 2A shows formation of a monoclonal cell cluster, where the cells express nuclear-localized RFP and were imaged using the red fluorescence channel. An advantage of fluorescent focusing over brightfield is that debris, which is sometimes present in cell culture plates, typically does not fluoresce, reducing the number of false negatives. The eSight software can easily enumerate the number of red cells, plotting them as a function of time based on red object count. Figure 2A shows a colony arising from a single cell, while 2B shows the number of red cells as a function of time.

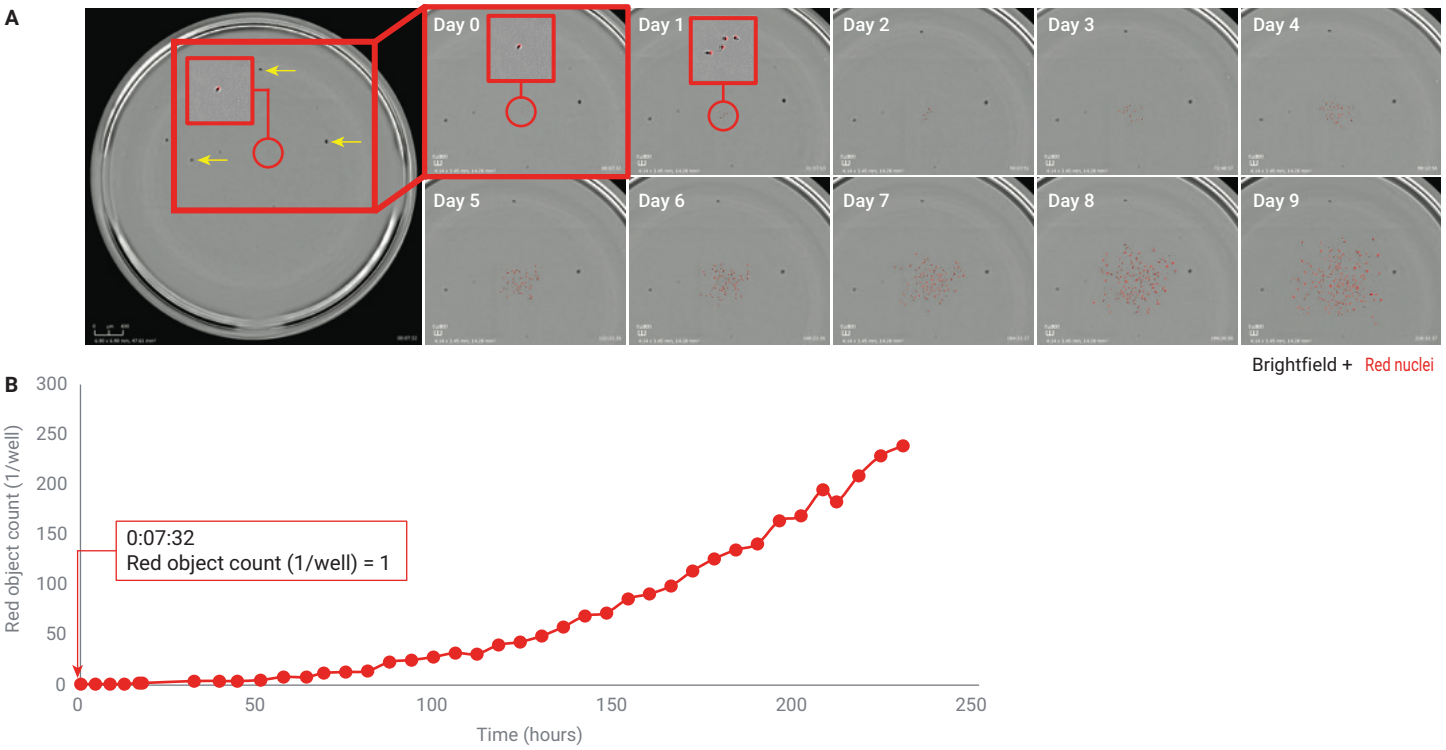

Figure 2. Tracking and monitoring of monoclonal colony formation using the Dilution Cloning module of the Agilent RTCA eSight system. (A) Fluorescence and brightfield merged images of a well containing a monoclonal colony. Debris is indicated by yellow arrows. Red boxes in Day 0 and Day 1 show enlarged versions of the corresponding area indicated by the smaller boxes. Day 0 shows a single red fluorescent cell was deposited in this well. (B) The red object count as a function of time for the well shown in Figure 2A, as determined by the eSight software. The software verifies that the well initially contained a single fluorescent object, demonstrating that the colony arose from a single cell.

Conclusion

This technical overview presents the combination of a limiting dilution method with an Agilent xCELLigence RTCA eSight system to screen and identify monoclonal cell lines. Limiting dilution, which is simple, repeatable, and gentle on cells, presents an advantage over other methods. The eSight automatically collects and analyzes images, generating cell growth curves based on various parameters (object count, area, and confluence) using brightfield and fluorescence channels, enabling the identification of monoclonal cell lines. Combining automated live cell imaging with limiting dilution preserves the advantages, while greatly reducing labor, time, and cost associated with manual screening to verify monoclonality. Additionally, images obtained can be used for submissions to regulatory agencies.

Note that this approach requires optimization and appropriate care. This includes use of an appropriate dilution ratio to obtain a higher number of wells with single cells, keeping the bottom of the plate clean, and filtering the culture media with a 0.22 µm filter (after adding FBS) to reduce debris. Debris can sometimes be identified as cells, resulting in false negatives. Potential issues are easily avoided when best practices are followed. Further reading (a troubleshooting guide) is included in the appendix to provide additional guidance. In conclusion, this study shows that the limited dilution method, in combination with the eSight system, results in a faster and more reliable workflow to develop monoclonal cell lines.

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Appendix

Dilution cloning troubleshooting guide

The technical overview *Limiting Dilution Method Using the Agilent xCELLigence RTCA eSight System* describes a combination of a traditional, useful method used to isolate monoclonal stable cell lines with xCELLigence eSight technology. Overall, monoclonal cell lines and image evidence can easily be obtained using the live cell imaging system combined with limiting dilution cloning. In particular, the solid automatic single cell focusing capability, whole well imaging, and real-time analysis capability of the xCELLigence RTCA eSight system increases speed and reliability in Dilution Cloning Assays. However, some challenges may pose an issue with the Dilution Cloning module and brightfield focusing. Should users encounter such challenges, additional suggestions and tips for assay optimization are provided below.

1. Brightfield imaging of the cells settling to the edge of the well can be challenging due to lower lighting (and tends to be a common occurrence/issue for most imaging systems). The lighting on the edge of the well is affected by the well walls and the meniscus of the medium. As an example, in Figure 3A, the Brightfield focusing does not adequately focus the cell on the edge of the well on Day 1.
2. Cell-sized debris disturbs the recognition of the single cell on Day 0 through brightfield (Figure 4A). The recognized "cell" was debris, and the real cell is not recognized.
3. Debris or fibers at the bottom of the well or in the medium may disturb the brightfield focusing (Figure 4B).

In such cases (2 and 3, as mentioned above), it is helpful to keep the bottom of the plate clean and filter the culture media with a 0.22 µm filter before experimenting.

In contrast, fluorescence focusing is less susceptible to the interference of cell debris and more accurately captures single cells (Figures 3B and Figure 4C). Therefore, fluorescence focusing is preferred when fluorescence labeling or intrinsic fluorescence is available. If fluorescence is not available, increasing manual inspection or a second round of dilution cloning may be necessary due to certain challenges of brightfield imaging, as described here.

Overall, this study shows that the limited dilution method, in conjunction with the RTCA eSight system, results in a significantly faster, data-rich, and more reliable workflow for the development of monoclonal cell lines.

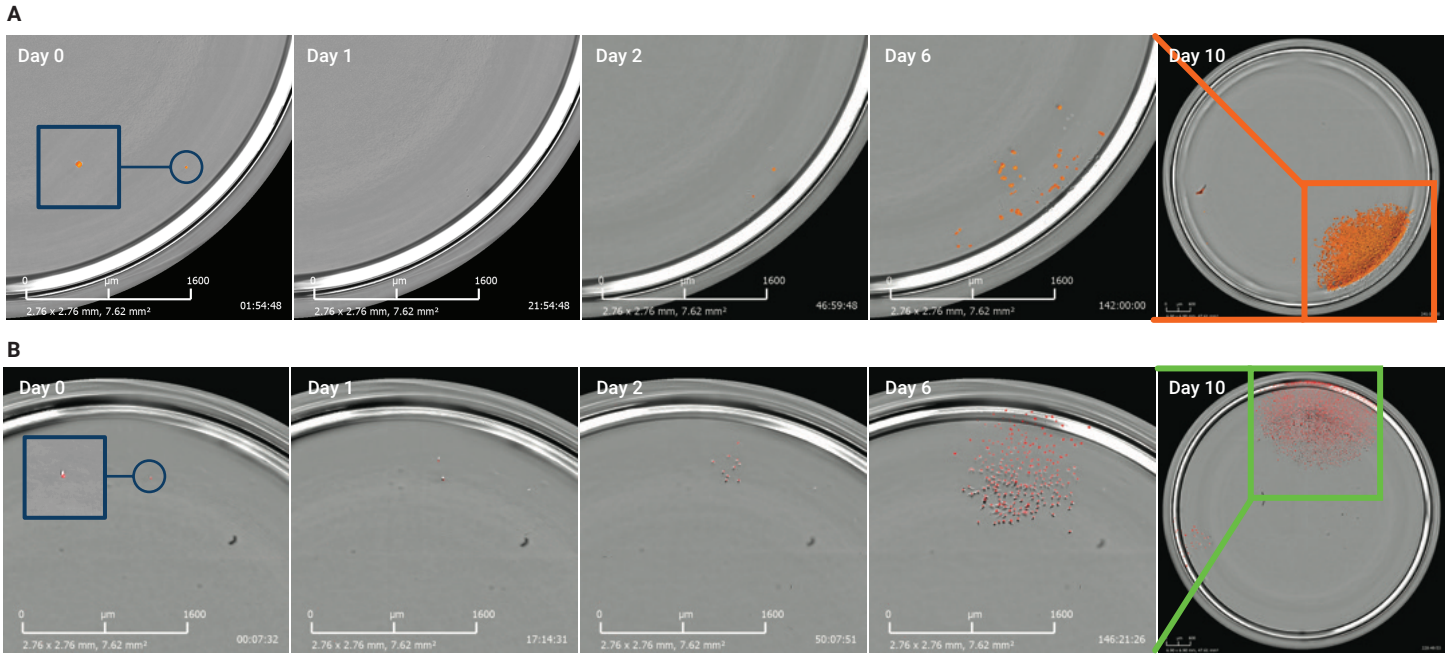

Figure 3. Representative images showing focus and recognition of cells at the edge of the well using brightfield or fluorescence focusing in dilution cloning assays. (A) Brightfield focusing can pose focusing challenges at the edge of the well, where the cell is not detected in the Day 1 image. (B) Fluorescence focusing helps to overcome these challenges. The cell on Day 0 is highlighted and enlarged within the blue box.

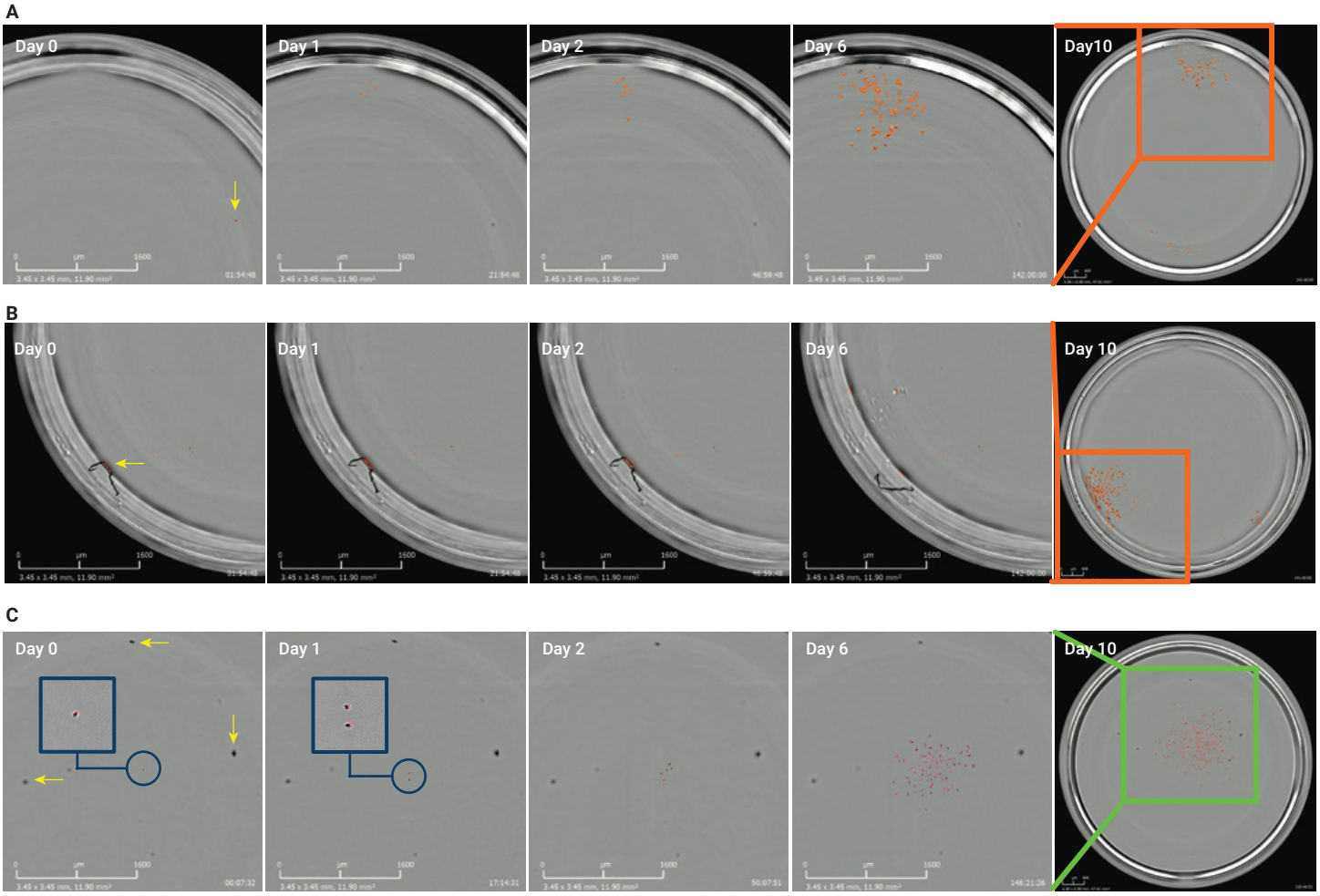

Figure 4. Representative images showing the focus and recognition of cells in the presence of debris/fibers using brightfield or fluorescence focusing in dilution cloning assays. (A) and (B) When using brightfield focusing, debris/fibers are brought into focus, and cells may become blurred. (C) Robust fluorescence focusing is more robust and less susceptible to errors associated with debris. The cell at Day 0 is highlighted and enlarged within the blue box. Debris/fibers are indicated by the yellow arrows.

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