APPLICATION NOTE

Non-disruptive quantification of 2° reprogrammed iPS colonies using Celigo imaging cytometer.

Introduction

Current methodologies for the detection of induced Pluripotent Stem Cell (iPSC) reprogramming are either disruptive (flow cytometry) or low in throughput (fluorescent microscopy). Using the Celigo® Imaging Cytometer and secondary iPS reprogramming, we have developed a methodology that combines the advantages of both flow cytometry and fluorescent microscopy. This approach is based on the fluorescent identification of iPSC colonies that express the four reprogramming factors: Oct4, Sox2, Klf4 and c-Myc, by expression of mOrange placed after the four factors following internal ribosome entry site (IRES) and the progress of reprogramming using fluorescent detection of the pluripotency reporter Nanog-GFP+ cells within these colonies. This method can be used to not only follow the reprogramming kinetics, but could also be used to examine the effect of extrinsic factors, thus, providing a strong tool to investigate molecular mechanisms of reprogramming.

Nexcelom's Celigo imaging cytometer has been applied to provide automated, rapid assessment of iPSC reprogramming. Using f-theta optics, Celigo provides high quality, whole well images using brightfield and/or fluorescent illumination. Automated segmentation and analysis provides quantitative output of iPSC reprogramming based on mOrange and GFP fluorescent colony detection.

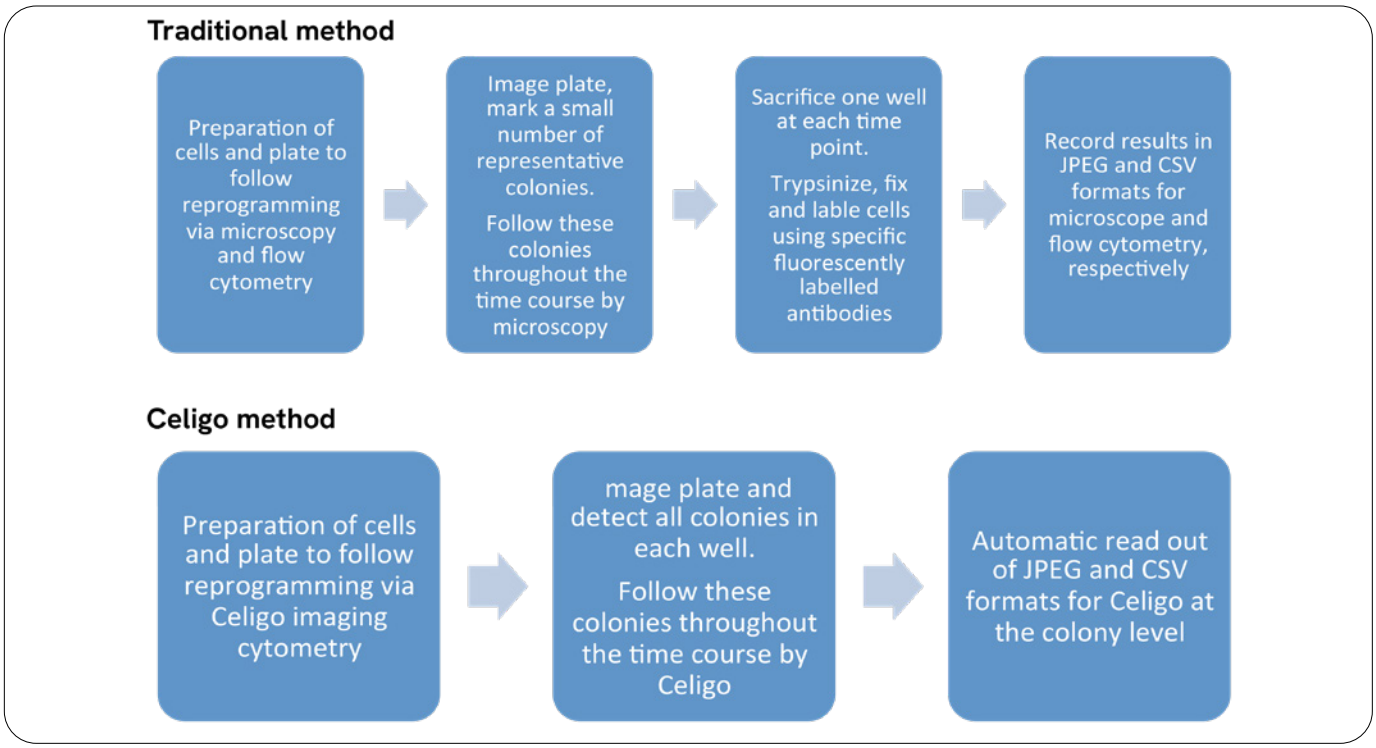
Celigo provides several key benefits:

- High throughput whole well imaging – e.g. Two channel/ two color 7-minute read time for a 6-well plate
- Three fluorescent channels (blue, green, red) and brightfield imaging
- Objective segmentation and automated quantification of colony numbers
- High resolution images, equivalent to 10-15x objective microscopy
- Automated sample analysis reduces time, labor and variability

Materials and methods

Traditional vs. Celigo approach

The Celigo method combines the qualitative, non-disruptive imaging properties of microscope-based analysis with the higher throughput, quantitative properties of flow cytometry analysis in one straightforward assay. More specifically, the Celigo method allows whole well colony enumeration giving the user statistical confidence in their reprogramming experiment with an amenable read time of 7 minutes. Furthermore, the single plate analysis reduces the high cell burden needed with FC analysis and the user can visualize heterogeneity of reprogramming per colony and track this throughout the time course.



Secondary reprogramming assay

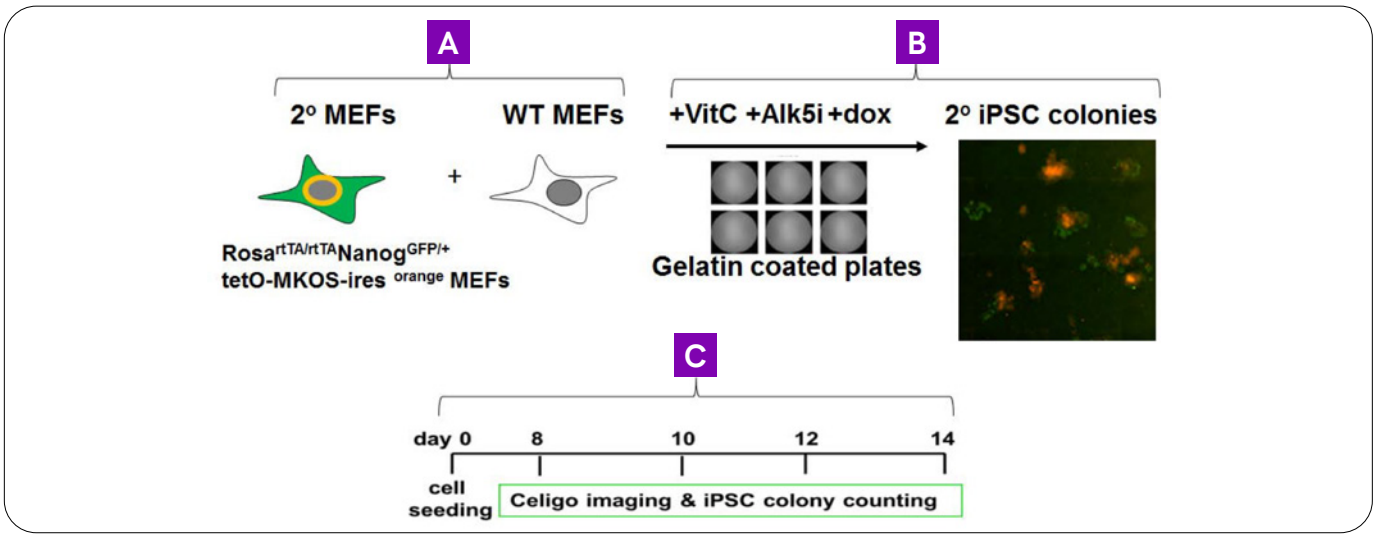


Figure 2. A) Transgenic Nanog GFP MEFs were diluted to 30% by addition of wild type 129 MEFs. B) They are plated in a gelatinized 6-well plate at 1×10^5 cells per well. Cells were cultured in reprogramming medium, GMEM. Medium was changed every 2 days. C) iPSC colonies were imaged and counted on the Celigo every two days from days 8 through 14.

Celigo reprogramming

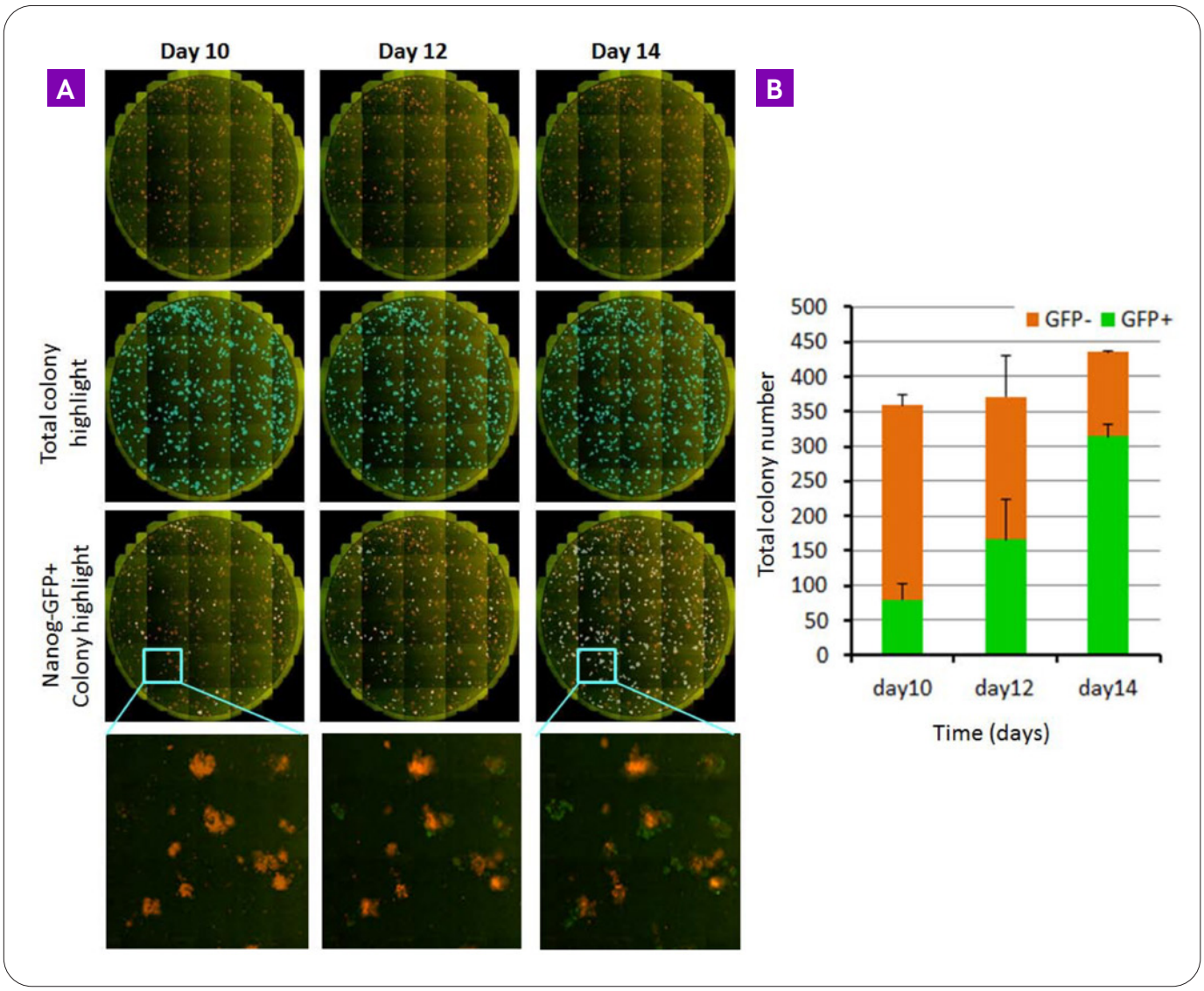


Figure 3. A) Typical thumbnail images of wells at days 10-14 of reprogramming (top), total colony number and Nanog GFP+ colonies highlighted with an overlay option. Bottom: high magnification of images from the blue square. B) Graphical analysis of reprogramming using Nanog-GFP+ expressing and mOrange+ MKOS colonies.

- Detection and enumeration of total, mOrange+ MKOS and Nanog-GFP colonies are simple and easily visualized with the overlay option, see above.
- Experimental settings are stored to make reanalysis of time points simple.
- Simple method for tracking reprogramming provides the user with a tool to investigate the molecular mechanism of reprogramming e.g. shRNA, growth factors, etc.

shRNA and its effect on reprogramming

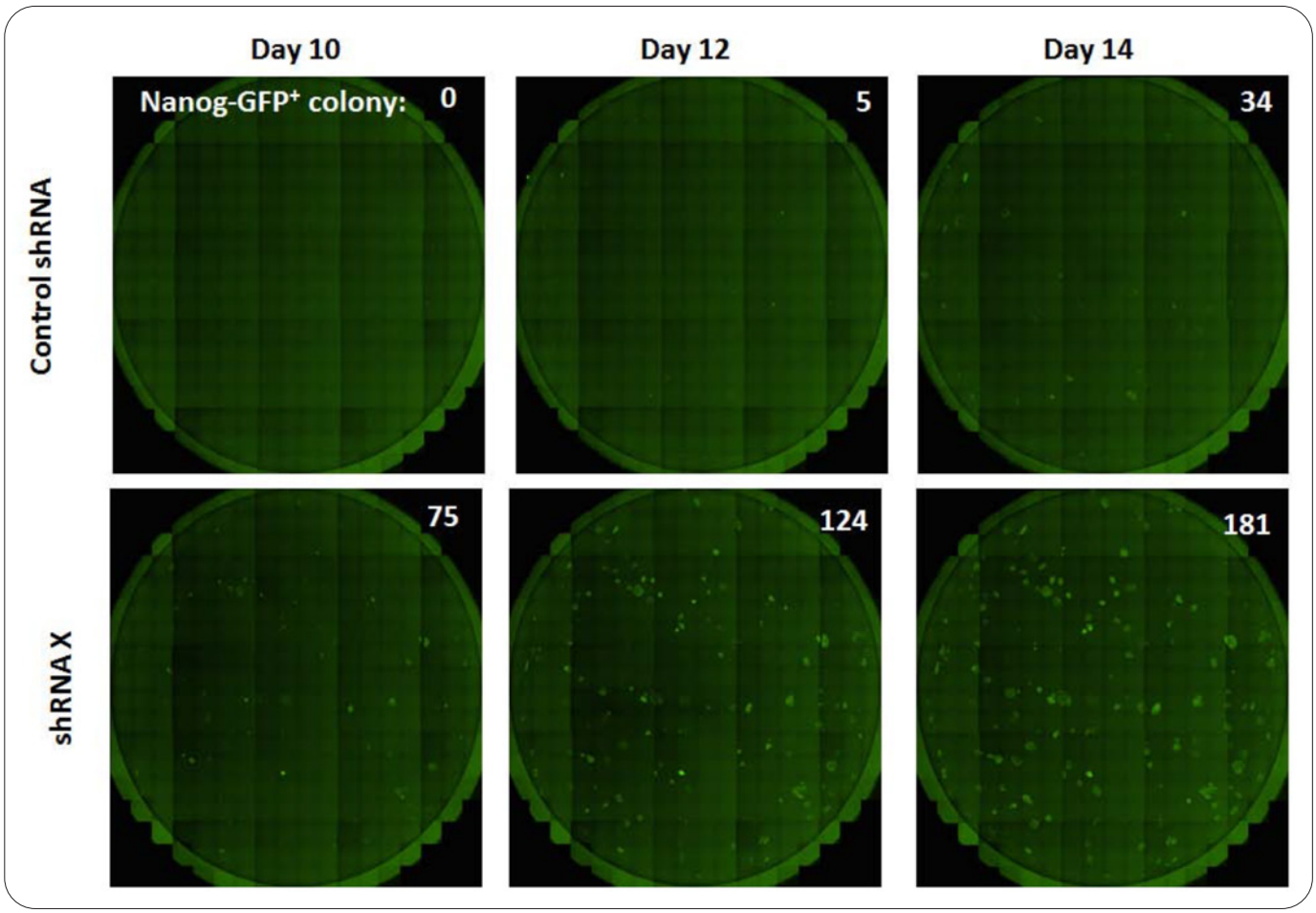


Figure 4. Typical thumbnail images of Nanog GFP+ colonies and their counts from wells at days 10, 12 and 14 of reprogramming with a control shRNA (top) or shRNA X (bottom).

- The results demonstrate that shRNA X increases the rate of reprogramming, suggesting that the gene is hampering reprogramming, and the elimination of this roadblock is important for efficient and fast reprogramming.
- Celigo allows objective and consistent quantification of colony numbers.
- A fast scanning speed allows imaging of the same plate throughout the time course without disrupting reprogramming.
- Nanog GFP colony detection alone can also be used as a method to track reprogramming efficiency.

Celigo reprogramming scaled down

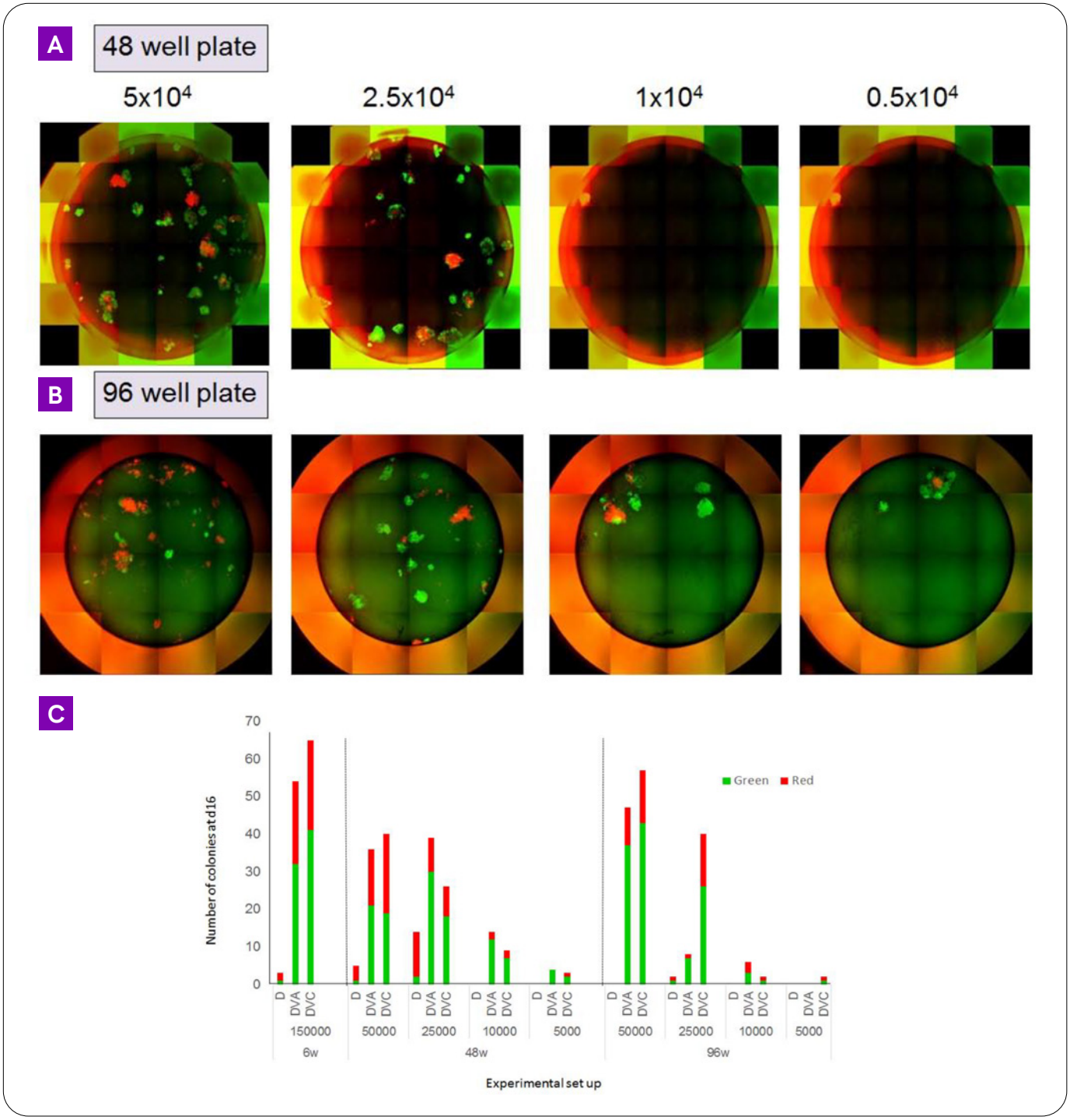


Figure 5. A) Titration of Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS-ires ^{orange} MEFs and the effect on colony production in a 48-well plate at day 16 of reprogramming. Typical thumbnail images of wells containing 5, 2.5, 1 and 0.5 x 10^4 Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires ^{orange} MEFs (top). B) Titration of Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires ^{orange} MEFs and the effect on colony production in a 96-well plate at day 16 of reprogramming. Typical thumbnail images of wells containing 5, 2.5, 1 and 0.5 x 10^4 Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires ^{orange} MEFs (top). C) Graphical analysis of scaled down reprogramming at day 16 using Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires ^{orange} MEFs.

- Objective, fast colony counting with Celigo allows for the scaling down ofreprogramming experiments.
- 150,000 Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ^{orange} MEFs in combination with the necessary number of WT MEFS produce an over confluent well, thus 5 x10^4 Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ^{orange} MEFs were used in 48- or 96-well plates.
- Image and graphical data demonstrate that reprogramming experiments can be scaled down and performed in either a 48- or 96-well plates.

Conclusions

The **Celigo® Imaging cytometer** is a bench top *in situ* cellular analysis system that rapidly provides high integrity whole well images for routine brightfield and fluorescent cellular analysis with a simple workflow.

- Nexcelom's Celigo imaging cytometer provides automated, rapid assessment of iPSC reprogramming.
- Additional applications can be used to aid and characterize differentiation procedures, for example the Embryoid Body (EB) Application can enumerate and characterize EBs on size, a contributing factor to efficient differentiation.
- Detection of pluripotent and differentiation markers can also be detected using the Expression Analysis and Confluence applications [1-3].
- User-friendly and intuitive software allows those with little imaging experience to generate valuable data.

References

1. Sproul, A.A., et al., Characterization and molecular profiling of PSEN1 familial Alzheimer's disease iPSC-derived neural progenitors. *PLoS One,* 2014. 9(1): p. e84547.
2. Hua, H., et al., iPSC-derived beta cells model diabetes due to glucokinase deficiency. *J Clin Invest*, 2013. 123(7): p. 3146-53.
3. Golipour, A., et al., A late transition in somatic cell reprogramming requires regulators distinct from the pluripotency network. *Cell Stem Cell*, 2012. 11(6): p. 769-82.

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