Non-disruptively count and quantify fluorescent iPS colonies during 2° reprogramming: 7 min per 6-well plate, dual-fluorescence whole well imaging cytometry

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1. ABSTRACT

Current methodologies for the detection of inducible Pluripotent Stem (iPS) reprogramming are either disruptive e.g. flow cytometry (FC) or low in throughput e.g. fluorescent microscopy (FM). Using the Celigo S 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 iPS colonies that express the four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc, by expression of mOrange placed after the four factors following 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.

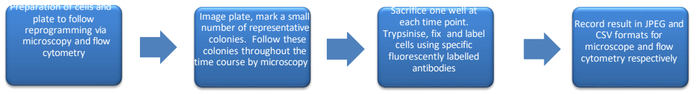
Revvity's Celigo S imaging cytometer has been applied to provide automated, rapid assessment of iPS reprogramming . Using f-theta optics, Celigo provides high quality, whole well images using bright field and/or fluorescent illumination. Automated segmentation and analysis provides quantitative output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

Celigo provides several key benefits;

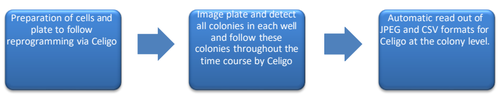
- High throughput whole well imaging – e.g. Two channel/ two colour 7 min read time for a 6 well plate
- Three fluorescent channels (blue, green, red) and bright field imaging
- Objective segmentation and automated quantification of colony numbers
- High resolution images equivalent to 10-15x objective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

2. TRADITIONAL vs CELIGO ASSAY PROTOCOL

Traditional method

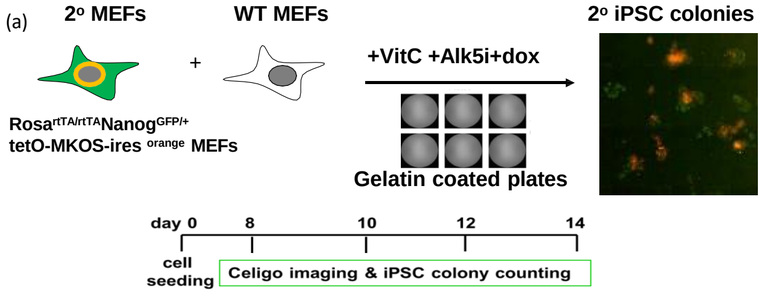


Celigo method



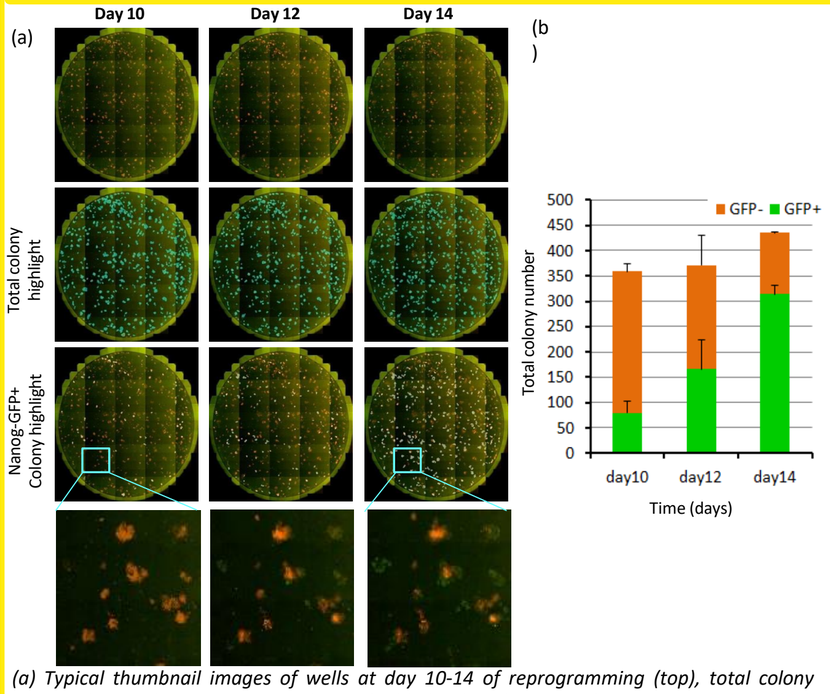
The Celigo method combines the qualitative, non disruptive imaging properties of the microscope based analysis with the higher throughput, quantitative properties of the flow cytometery analysis in one easy to perform assay. More specifically the Celigo method allows whole well colony enumeration giving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

3. SECONDARY REPROGRAMMING ASSAY PROTOCOL



(a) Transgenic Nanog GFP MEFs were diluted to 30% by addition of wild type 129 MEFs and plated in gelatinized 6 well-plate at 1×105 cells per well (30,000 Tg). Cells were cultured in reprogramming medium, GMEM, 10% FBS, penicillin-streptomycin, 1× Non-Essential Amino Acids (Invitrogen), 1mM Sodium Pyruvate, 0.05 mM 2-Mercaptoethanol, 1000 U ml−1 Leukemia inhibiting factor (LIF), dox (300 ng ml−1), Vitamin C (10 µg ml−1) and Alki (500 nM). Medium was changed every 2 days. iPS colonies were imaged and counted on the Celigo every two days from d8-14.

4. CELIGO REPROGRAMMING

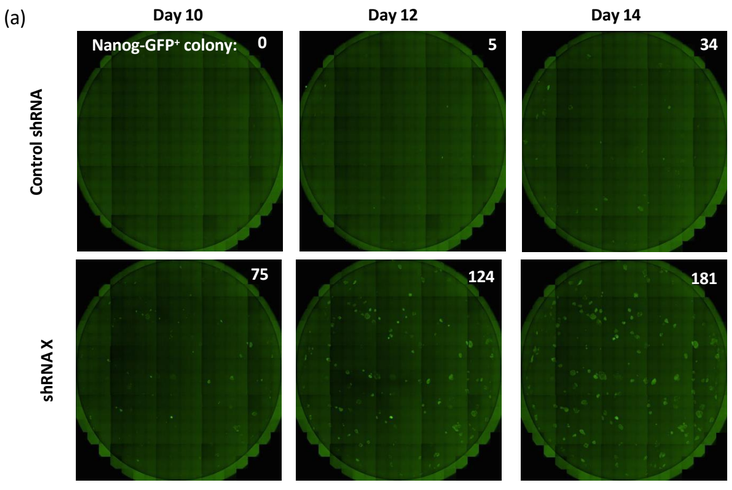


(a) Typical thumbnail images of wells at day 10-14 of reprogramming (top), total colony number and Nanog GFP+ colonies highlighted with an overlay option. Bottom: high magnification of images of the from the blue square

(b) Graphical analysis of reprogramming using Nanog-GFP + expressing and mOrange+ MKOS colonies

- Detection and easy enumeration of total, mOrange+ MKOS and Nanog-GFP colonies is simple and easy to visualise with the overlay option, see above.
- Saved experimental settings make reanalysis of time points in reprogramming 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.

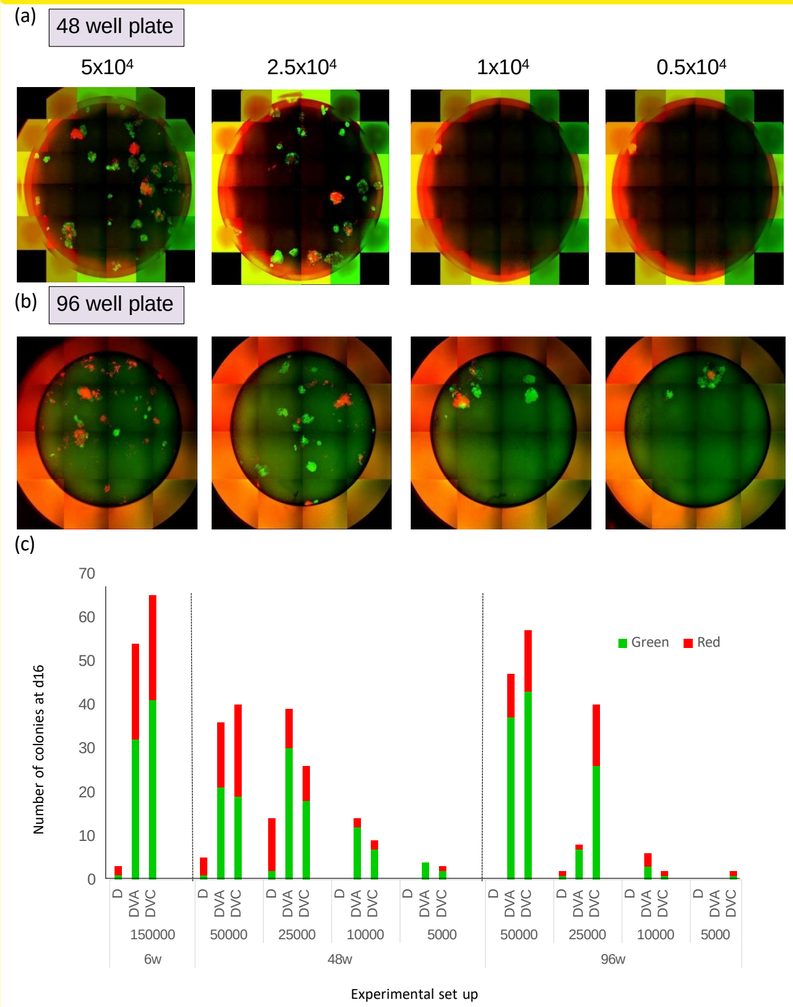
5. shRNA and ITS EFFECT ON REPROGRAMMING



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

- The results demonstrate that shRNA X increases the 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.
- Fast scanning speed allow 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

6. CELIGO REPROGRAMMING SCALE DOWN



(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 scale down reprogramming at d16 using Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires ^{orange} MEFs

- Objective, fast colony counting with Celigo allows scaling down reprogramming experiments
- 150000 Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ^{orange} MEFs in combination with the necessary number of WT MEFS produce an over confluent well thus 5 x104 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 plate.

7. CONCLUSION

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

- Revvity's Celigo imaging cytometer has been applied to provide automated, rapid assessment of iPS reprogramming.
- Addition applications can be used to aid and characterise differentiation procedures e.g. Embryoid body (EB) application can enumerate and characterise EBs on size; a known contributing factor in efficient differentiation.
- Detection of pluripotent and differentiation markers can also be detected using the expression analysis and confluence applications.(1, 2, 3)
- User-friendly and intuitive software allows even those with little imaging experience to generate valuable data.

References

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3. Azadeh Golipour, Laurent David, Jeffrey L. Wrana, et al (2012)A Late Transition in Somatic Cell Reprogramming Requires Regulators Distinct from the Pluripotency Network. Cell Stem Cell (11), 769–782.