# A rapid fluorescent image cytometry method for validation and monitoring of 2° reprogrammed iPSC colonies

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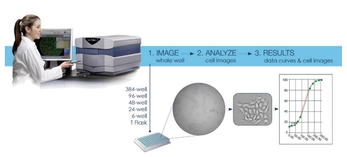
## 1. ABSTRACT

Flow cytometry (FC) and fluorescent microscopy (FM) have been commonly used for the detection of induced Pluripotent Stem Cell (iPSC) reprogramming, which often have limitations, where flow cytometry requires disruption of adherent iPSCs by trypsinization, and fluorescent microscopy requires manual qualitative analysis that is low throughput. In the recent years, image-based cytometry systems have been utilized to perform direct whole well cell-based assays in microplates without trypsinization and with comparable sensitivity as current fluorescence detection methods. In this work, we developed an automated method for detection of iPSC colonies utilizing the combination of the Celigo S Imaging Cytometer and secondary iPSC reprogramming. This approach is based on the fluorescent identification of iPSC colonies that express the four reprogramming factors, Oct4, Sox2, Klf4 and c-Myc expressing mOrange following ires. The reprogramming progress is also monitored using fluorescent detection of the pluripotency reporter Nanog-GFP$^{+}$ cells within these colonies. Results demonstrated the capability of the imaging cytometer showing the increase in Nanog-GFP+/mOrange+ iPSC colonies in respect to time and also increase in iPSC colonies when treated with shRNA X. The Celigo Imaging Cytometer allowed high throughput whole well fluorescence imaging and analysis of the iPSC colonies, which provided accurate direct measurement of colony numbers as well as improve research efficiency through automation. 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.

## 2. CELIGO IMAGING CYTOMETRY FOR SECONDARY REPROGRAMMING VALIDATION




1. Celigo Imaging Cytometer is a plate-based cytometer that can scan the entire well of standard microplates and captures bright-field and fluorescent images
2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity
3. The measured parameters are used to generate cell proliferation kinetic data, GFP/RFP expression, tumor spheroid size change, DNA cell cycle analysis, apoptosis, and ADCC cytotoxicity results

## 3. TRADITIONAL VS CELIGO ASSAY PROTOCOL

**Traditional method**

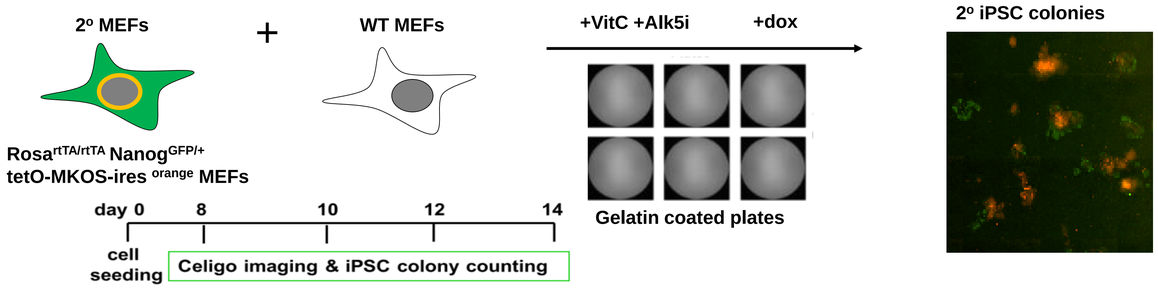
Preparation of cells and plate to follow reprogramming via microscopy and flow cytometry → Image plate, mark a small number of representative colonies. Follow these colonies throughout the time course by microscopy → Sacrifice one well at each time point. Trypsinize, fix and label cells using specific fluorescently labelled antibodies → Record result in JPEG and CSV formats for microscope and flow cytometry respectively

**Celigo method**

Preparation of cells and plate to follow reprogramming via Celigo → Image plate and detect all colonies in each well and follow these colonies throughout the time course by Celigo → Automatic read out of JPEG and CSV formats for Celigo at the colony level.

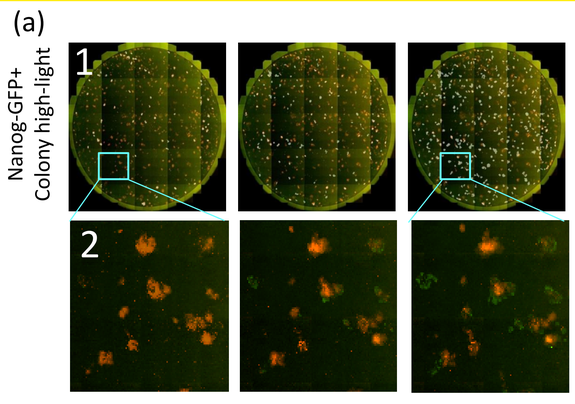
- The Celigo method utilizes the qualitative and non-disruptive microscope-based imaging characteristic for higher throughput and quantitative image cytometric analysis
- Celigo allows whole well colony enumeration for standard reprogramming experiment in less than 7 minutes
- Single plate anlaysis reduces the high cell number required with FC analysis
- User can visualize heterogeneity of reprogramming per colony and track this throughout the time course

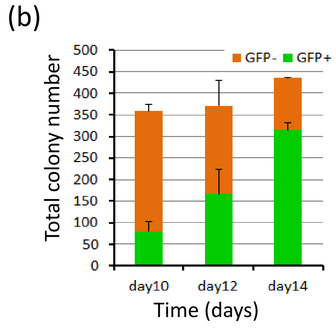
## 4. SECONDARY REPROGRAMMING ASSAY PROTOCOL




1. Transgenic Nanog GFP MEFs were diluted to 30% by addition of wild type 129 MEFs and plated in gelatinized 6 well-plate at 1×10$^5$ cells/well (30,000 Tg )
2. 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.
3. iPS colonies were imaged and counted on the Celigo every two days from day 8-14.

## 5. DETECTION OF MORANGE+, MKOS, AND NANOG-GFP COLONIES USING CELIGO



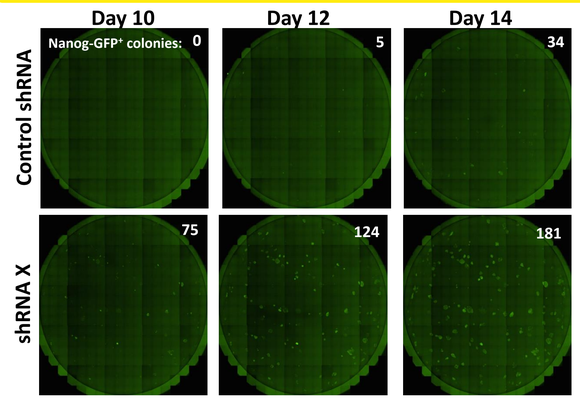




- Celigo can be used for tracking reprogramming such as shRNA, growth factors, etc.

a) Images of wells at day 10-14 of reprogramming (1). High magnification of images from the blue squares (2)

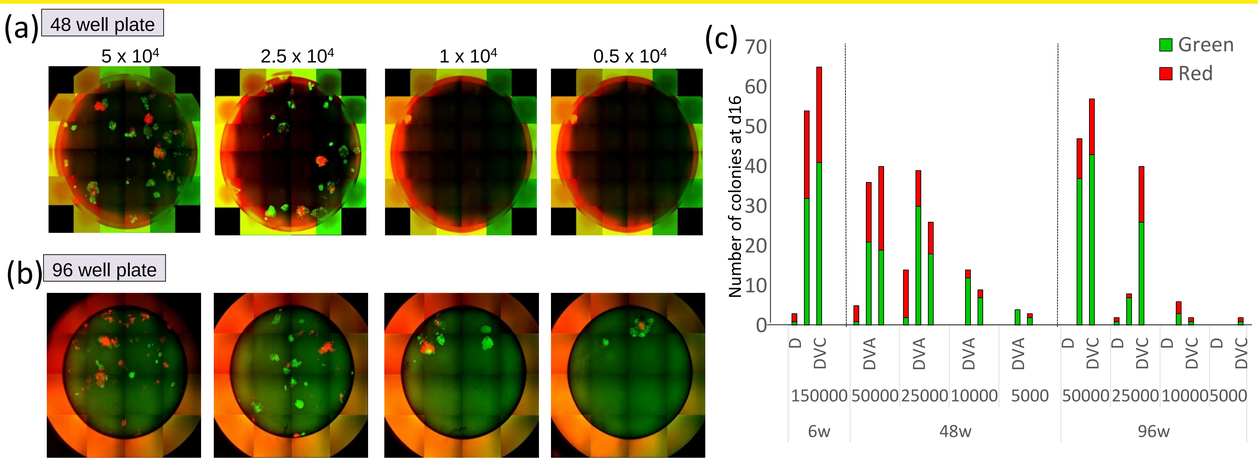
b) Reprogramming results using Nanog-GFP + expressing and mOrange+ MKOS colonies

## 6. shRNA AND ITS EFFECT ON REPROGRAMMING




- Fluorescent 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

## 7. 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 at 5, 2.5, 1 and 0.5 x 10$^4$ Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires$^{orange}$ MEFs

(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 at 5, 2.5, 1 and 0.5 x 10$^4$ Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires $^{orange}$ MEFs

(c) Results of scale down reprogramming at day 16 using Rosa$^{rtTA/rtTA}$ Nanog$^{GFP/+}$ MKOS ires $^{orange}$ MEFs

- 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 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 plate

## 8. SUMMARY AND CONCLUSION

- Nexcelom's Celigo imaging cytometer demonstrated automated, rapid assessment of iPS reprogramming
- Other applications can be used to characterize differentiation procedures
  - Embryoid body (EB) application can enumerate and characterize 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**

1. Sproul AA, Jacob S, Pre D, Kim SH, Nestor MW, et al. (2014) Characterization and Molecular Profiling of PSEN1 Familial Alzheimer's Disease iPSC-Derived Neural Progenitors. PLoS ONE 9(1): e84547.

2. Haiqing Hua, Linshan Shang, Hector Martinez, et al ( 2014) iPSC-derived β cells model diabetes due to glucokinase deficiency. *J Clin Invest.* 2013;123(7):3146–3153.

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.