Live cell quantification using image analysis

Minjung Song, Iman Mali, Venkat Pisupati, Florian T Merkle

Wellcome-MRC Institute of Metabolic Science, University of Cambridge, Cambridge, CB2 0QQ, United Kingdom

Purpose

This protocol describes image-based quantification of human pluripotent stem cells (hPSCs), which could be adapted for other cell populations that grow in a monolayer. It utilises a membrane-bound stain and requires a high-content imaging system such as a PerkinElmer Oprea Phenix or similar and downstream image analysis pipelines. The suggested dye labels live cells, fluoresces in far red, and shows no evidence of toxicity in hPSCs. This protocol is useful in situations where cell quantification by manual or automated counting becomes rate-limiting, such as when multiple lines are grown high-throughput differentiation experiments on different genetic backgrounds, or for survival or morphology-based screens in systems where cells have clearly defined cell borders.

Materials & Software

Item	Catalog #	Vendor
CellMask Deep Red plasma membrane	C10046	ThermoFisher Scientific
Dulbecco's phosphate buffered saline containing calcium and magnesium (DPBS +/+)	14040133	ThermoFisher Scientific
µ-Plate 24 Well Black	82426	Ibidi
StemFlex	A3349401	ThermoFisher Scientific
Microseal B seals	MSB1001	Bio-Rad
Harmony	HH17000010	PerkinElmer

Media and Reagents

staining solution (12 ml)

Name	Volume
CellMask Deep Red plasma membrane dye	12 µl
Culture media (e.g. StemFlex)	12 ml

Cell culture and staining

Cell plating

1. Coat plate with appropriate substrate to prepare cells for plating. We have found the 24-well black µ-Plate from Ibidi allows good cell growth and is compatible with high-content imaging systems.
2. When plating cells for culture, ensure that they are evenly distributed in the culture well by using a large enough volume of medium (e.g. 500 ml per well of a 24-well plate) and shaking the plate left-to-right and top-to-bottom, avoiding circular motions that might cause cells to gather in the center. Repeat these motions once

the plate has been transferred to the incubator shelf and avoid vibrations by gently closing the incubator, and ensure incubator shelves are level.

Cell staining

3. Once cells have reached approximately 80% confluence, aspirate medium, and wash once with DPBS. Add a standard volume of staining solution (e.g. 500 ul for a well of a 24-well plate) to label the plasma membrane. [**Note:** the 1:1000 dilution was optimised with hPSCs at approximately 80% confluence, but may need to be optimised for other cell types or densities.]
4. Incubate cells for 10 min at 37°C.
5. Wash cells two times with DPBS. (For a 24-well plate, 500 µl of staining solution per well was applied).
6. After final washing, add culture media. Plates can be imaged immediately, or returned to the incubator and imaged within 2 hours of staining.

Imaging

To image plates, we recommend an automated confocal imaging system such as the PerkinElmer OperaPhenix. Enter the plate parameters and define regions to be imaged ahead of time. The exact magnification and number of fields to be chosen will depend on the needs of the user, but a good place to start is 20x magnification in a 24 well plate, and 10-30 randomly selected fields sampling representative regions of the plate and covering at least 5% of its surface area.

1. Seal the wells of the plates with sterile B seal film and bring the plate to the imaging facility. While wearing gloves, carefully wipe the bottom of the plate with a clean tissue to ensure that no dust, fingerprints, or media interfere with imaging.
2. For imaging cells stained with CellMask Deep Red plasma membrane dye, use a 647 nm laser at approximately 50% power setting and an exposure time of approximately 200 ms, to be optimised by the user. We have found it helpful to take a stack of 3 optical sections separated by 0.8 microns and starting at the surface of the plate where cells are attached with 20x Water objective. The dye stains the plasma membrane, and the images should clearly reveal the cell borders so that images can be readily segmented for quantification.
3. Image all wells to be quantified. It should be possible to image the entire plate in less than 30 minutes, in which case it can be performed at room temperature.
4. Return the plate to a biosafety cabinet after wiping it well with 70% ethanol. Carefully remove B seal film and return the plate to the incubator while awaiting results of image quantification.

Image analysis

Image segmentation and cell quantification can be performed with a variety of software platforms. We used Harmony, PerkinElmer's high-content imaging and analysis software.

1. In input images, select basic flatfield correction and maximum projection. (For max projection analysis, make sure all z-planes are selected)
2. Invert maximum projected images.
3. Remove noise from the background (e.g. with a sliding parabola filter) with settings to be optimised by the user.
4. Segment cells to enable quantification. The exact parameters will depend on the cell type and density. For hESC quantification at approximately 80% confluence with Harmony, we found that the 'Find nuclei' function (method B and M) worked

well. Other parameters such as threshold, size and splitting sensitivity will need to be adjusted to refine the identification. [**Note**: Take care to test parameters on multiple fields and wells during optimisation.]

When establishing an image analysis pipeline, dissociate and quantify cells using a manual hemocytometer and/or automated cell counter to calibrate the results from image-based quantification.