Journal of Visualized Experiments

Measuring the confluence of iPSCs using an automated imaging system.

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61225R2
Full Title:	Measuring the confluence of iPSCs using an automated imaging system.
Section/Category:	JoVE Biology
Keywords:	Stem cell biology; Cellular biology; induced pluripotent stem cells; confluence; coating.
Corresponding Author:	Claudia Compagnucci Ospedale Pediatrico Bambino Gesu Rome, Rome ITALY
Corresponding Author's Institution:	Ospedale Pediatrico Bambino Gesu
Corresponding Author E-Mail:	claudia.compagnucci@gmail.com
Order of Authors:	Valentina Magliocca
	Maria Vinci
	Tiziana Persichini
	Franco Locatelli
	Marco Tartaglia
	Claudia Compagnucci
Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US$2,400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Rome, Italy

TITLE:

Measuring the Confluence of iPSCs using an Automated Imaging System

AUTHORS:

Valentina Magliocca[1,2], Maria Vinci[3], Tiziana Persichini[2], Franco Locatelli[3], Marco Tartaglia[1], Claudia Compagnucci[1]

1. Genetics and Rare Diseases Research Division, Ospedale Pediatrico Bambino Gesù, IRCCS, Rome, Italy
2. Department. of Science, University Roma Tre, Rome, Italy
3. Department of Onco-hematology, Gene and Cell Therapy, Ospedale Pediatrico Bambino Gesù, IRCCS, Rome, Italy

Corresponding
Author: Claudia Compagnucci
claudia.compagnucci@opbg.net

Email addresses:
Valentina Magliocca: valentina.magliocca@hotmail.it
Maria Vinci: maria.vinci@opbg.net
Tiziana Persichini: tpersichini@os.uniroma3.it
Franco Locatelli: franco.locatelli@opbg.net
Marco Tartaglia: marco.tartaglia@opbg.net

KEYWORDS:

Stem cell biology, cellular biology, induced pluripotent stem cells, confluence, coating

SUMMARY:

The goal of the protocol is to compare different extracellular matrix (ECM) coating conditions to assess how differential coating affects the growth rate of induced pluripotent stem cells (iPSCs). In particular, we aim to set up conditions to obtain optimal growth of iPSC cultures.

ABSTRACT:

This study focuses on understanding how growing iPSCs on different ECM coating substrates can affect cell confluence. A protocol to assess iPSC confluence in real time has been established without the need to count cells in single cell suspension to avoid any growth perturbation. A high-content image analysis system was used to assess iPCS confluence on 4 different ECMs over time in an automated manner. Different analysis settings were used to assess cell confluence of adherent iPSCs and only a slight difference (at 24 and 48 hours with laminin) has been observed whether a 60, 80 or 100% mask was applied. We also show that laminin lead to the best confluence compared to Matrigel, vitronectin and fibronectin.

INTRODUCTION:

Induced pluripotent stem cells (iPSCs) are obtained from somatic cells and can be differentiated

into different cell types. They are often used as a system to model disease pathogenesis or perform drug screening, and also offer the potential to be used in the context of personalized medicine. Since iPSCs have great potential, it is important to fully characterize them for use as a reliable model system. We previously showed the importance of growing iPSCs in a hypoxic environment as these cells rely on glycolysis and an aerobic environment can cause redox imbalance[1]. iPSCs are also vulnerable to other culture conditions, particularly the extracellular environment. Optimization of culture conditions is a key issue to keep them healthy and proliferating. A healthy iPSC culture will lead to healthy differentiated cells that generally are the endpoint of the model used to understand molecular, cellular and functional features of specific human disorders or cellular processes.

In this study, a simple protocol has been used to test the confluence of iPSCs using different coating conditions in separate wells. iPSCs require a feeder layer of murine embryonic fibroblasts (MEF) in order to properly attach, but the coexistence of iPSCs and MEF makes it difficult to perform analysis like RNA or protein extraction since two populations of cells are present. In order to avoid the feeder layer, different proteins belonging to the extracellular matrix (ECM) have been used to recreate the natural cell niche and to have feeder free iPSC culture. In particular, Matrigel is a solubilized basement membrane preparation extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, which is enriched in extracellular matrix proteins (i.e., laminin, collagen IV, heparan sulfate proteoglycans, entactin/nidogen, and growth factors)[2-3]. The other used coating conditions are instead purified proteins with known relevance in building the ECMs: laminin-521 is known to be secreted by human pluripotent stem cells (hPSCs) in the inner cell mass of the embryo and it is one of the most common laminins in the body after birth[4-11]; vitronectin is a xeno-free cell culture matrix known to support growth and differentiation of hPSC[12-16]; fibronectin is an ECM protein important for vertebrate development and the attachment and maintenance of embryonic stem cells in a pluripotent state[17-25]. Since different coating conditions are available, we compare them in terms of their effect on iPSCs' confluence.

PROTOCOL:

1. Coating 96 well plates

NOTE: Different coatings were tested in the same plate but separate wells (see **Supplemental File**).

1.1. Dilute the Matrigel 1:100 in DMEM. Add 100 µL per well to the 96 well plates and incubate for 1 h at room temperature. Following this, remove the solution and wash the wells with 1 mL of DMEM twice.

1.2. Dilute laminin (20 µg/mL, LN-521) in PBS (with calcium and magnesium). Add 100 µL to the well and incubate at 4 °C overnight. The following day perform two washes with DMEM before seeding the cells.

1.3. Dilute vitronectin (10 µg/mL) in dilution buffer. Add 100 µL per well to the 96 well plate and incubate for 1 hour at room temperature. Wash the wells with PBS (without calcium and magnesium) before plating the cells.

1.4. Dilute HU-Fibronectin (30 µg/mL) in ddH_2O. Add 100 µL to the wells and incubate at room temperature for 45 minutes. Following this, wash the wells with the medium before seeding the cells.

2. Maintenance of iPSCs in culture

NOTE: iPSCs were purchased commercially. The iPSCs were derived from human fibroblasts and reprogrammed using episomal technology.

2.1. From the -80 °C freezer or liquid nitrogen, thaw the cryopreserved iPSCs in a 37 °C water bath. Clean the vial containing the cells with 70% ethanol prior to moving it into the biological safety cabinet.

2.2. Add the cell suspension to 5 mL of pre-warmed cell culture media (e.g., mTeSR1) drop by drop with a 1000 µL pipette in a 15 mL sterile conical tube.

2.3. Centrifuge the cells at 304 x *g* for 5 min at room temperature (RT).

2.4. Remove the media and resuspend the cell pellet in 4 mL of cell culture medium.

2.5. Plate the cell suspension into two wells of the 6 well plates (10^5 iPSCs per 6-well cell culture dish), where the mouse embryonic fibroblasts (MEFs) have been plated previously. Seed MEFs two days before plating iPSCs at a density of 2.4 x $10^4/cm^2$ in DMEM (containing 10% Fetal Bovine Serum, 1% L-glutamine and 1% Penicillin-Streptomycin).

2.6. After seeding, supplement the cell media with 10 µM of ROCK inhibitor Y-27632.

2.7. Grow the iPSCs on MEFs for the first 4-5 weeks and then in feeder free condition (MEF free condition), using one of the coating of interest (see step 1 and **Table 1**) in mTeSR1.

2.8. When the iPSCs are 70-80% confluent, passage 1:4 using 0.5 mM EDTA treatment for 3-5 min at RT. Add 1 mL of 0.5 mM EDTA for a 6 well plate (or proportional quantities for other types of plates). Leave the reagent to act for 3-5 min at RT. Transfer to new wells in feeder-free conditions and incubate at 37 °C, 5% CO_2, 20% O_2.

2.9. Change the media with fresh mTeSR1 every day and split the cells every 2 days.

3. Characterization of cell confluence

3.1. Use 96 well plates for the experiments.

3.2. Seed 10,000 cells per well following at least 1 month of culturing in feeder free condition in order to be sure that the MEFs were not passaged. Use disposable counting slides to count the cells with the optical microscope.

3.3. Perform the experiments in triplicate. Therefore, test each seeding condition in three wells.

3.4. Perform automated image acquisition from day 1 following seeding using a cytometer in bright-field mode. Perform automated image acquisition every 24 h for 5 days. For detailed information on the experimental parameters, refer to the **Supplemental File**.

3.5. Use auto-contrast and auto-exposure to better visualize cells.

3.6. Set the analysis setting (see **Supplemental File**) for confluence analysis to apply a mask of 60%, 80% and 100% per well, in order to evaluate the changes in focus due to the light refraction at the border of the wells. Use the different mask analysis setting mentioned above to analyze the cell confluence at each time point.

4. Statistical analyses

4.1. Report quantitative results as means ± standard error of the mean (SEM).

4.2. For comparing overall differences of the different coating conditions, obtain data using the same samples and perform the Student's paired-sample t-test. *P* values less than 0.05 are considered statistically significant, and all reported *p*-values are two sided.

5. Characterization of the cytoskeletal microfilaments

5.1. Fix cells with 4% paraformaldehyde (4% PFA) in PBS for 10 min at RT, followed by two washes in PBS (10 min total).

5.2. Add 100 µL of blocking solution (composed by 5% BSA, 0.1% Triton in PBS) to each well for 1 h at RT.

5.3. Remove the blocking solution and wash the samples twice with PBS for 10 min.

5.4. Add 100 µL of the phalloidin-conjugate working solution per sample and incubate for 1 h at RT.

5.5. Wash cells twice with PBS (10 min at RT).

5.6. Stain nuclei with Hoechst 33342 diluted 1:10000 in PBS for 10 min at RT.

5.7. Remove the Hoechst solution and wash the cells twice with PBS for 10 min each time.

5.8. Wash the sample with H_2O and let dry under a chemical hood.

5.9. Add 100 µL of mounting media (i.e. PBS:glycerol, 1:1) to cover the cells and preserve fluorescence of samples.

5.10. Observe cell at Ex/Em 493/517 nm on a laser-scanning confocal microscope equipped with a white light laser (WLL) source and a 405 nm diode laser. Acquire sequential confocal images using a HC PLAPO 40x oil-immersion objective. Use the same laser power, beam splitters, filter settings, pinhole diameters and scan mode for all examined samples.

REPRESENTATIVE RESULTS:

In this study, we investigated iPSCs confluence when grown on different coating conditions. Using a cytometer, we were able to obtain readily informative results in triplicates in 5 days. Since iPSCs hardly attach to plastic vessels and a coating is necessary to support their proliferation, we decided to monitor the confluence of human iPSCs as it is indicative of the health of the cell culture and it may reflect on their differentiation potential. After in vitro expansion, we seeded the iPSCs on different ECM substrates and analyzed cells by observation of the sample images acquired in bright-field and using phalloidin staining (used for staining actin filaments, also known as F-actin) in order to understand their adhesion to the vessels (**Figure 1**). In fact, phalloidin staining allows visualization of the degree of cell adhesion to the surface of the vessel and therefore to the specific coating used for the vessel. Cells that are adherent to the coating showed clearly visible cytoskeletal microfilaments instead of collapsed microfilaments. The observation of the brightfield in combination with phalloidin staining document a good level of adhesion of the iPSCs to the coated surface.

To investigate the confluence, we seeded the iPSCs with Matrigel, LN-521, vitronectin and Hu-fibronectin in triplicates, and performed the experiment three times. In order to avoid the light refraction due to the edge of the well, we applied three types of analysis setting with a mask of 60, 80 and 100%, and observed that they are similar in picking the cells and avoiding the background (**Figure 2**). The results obtained show that iPSCs seeded on LN-521 show a high rate of cell proliferation in a linear fashion during time, comparing it with the other coatings and that these differences are statistically significant (asterisks in **Figure 3A-C**). Cells seeded on Matrigel, Vitronectin or Hu-Fibronectin show a linear proliferation rate in the first 96 hours but they also show an increased slope of the confluence curve in the last 24 hours (independently of the mask used, 60%, 80% or 100%, **Figure 3A-C**). Since the initial difference at 24 h for the different coatings can be due to differences in cell attachment, cell growth has been normalized to the 24 h for the later time points (from 48 to 120 h) (**Figure 3D-F**). The graphs obtained using the 60, 80 and 100% mask show that no differences exists in terms of confluence among the different coatings and that the differences observed with LN-521 are most probably due to an increased ability of the iPSCs to adhere to this coating when passaged.

Figure 1. Representative bright-field images and Phalloidin staining of iPSCs seeded on

different ECM coatings after 3 days. Bright-field images showing that the cells are healthy on the coating used and that they are well attached to the vessels as documented by the phalloidin staining showing clearly visible cytoskeletal microfilaments. Scale bar: 25 µm

Figure 2. Representative bright-field images showing three different analyses setting for the masks used to perform confluence analyses. Mosaic obtained with a cytometer using bright-field images (16 images/well from a 96 well plate). In green the analysis segmentation displays clearly the different mask applied (60, 80 100%) to avoid or include the round edge of the well. Scale bar: 500 µm.

Figure 3. Cell confluence analysis of iPSCs seeded on differently coated vessels. Graph representing the cell confluence of iPSCs seeded on differently coated vessels. Data were analyzed after acquisition with the appropriate software using a (**A**) 60% mask (**B**) 80% (**C**) 100% for 5 days (120 h). Normalization of the confluence of the 48 h to 120 h time points to the first time point (24 h) is shown in (**D, E, F**). The data were obtained from three independent experiments. Data are represented as mean ± SEM. n= 3 * $p<0.05$.

Table 1. List of coating compounds used to analyze the confluence. The name, initial and final concentration of different coatings used are reported*. The initial concentration of Matrigel is variable, depending on the batch.

DISCUSSION:

The use of iPSCs for disease modeling and future drug screening together with their possible application in precision medicine makes it a technology of great relevance and for this reason we believe that it is necessary to clearly understand the in vitro culturing condition that better resemble the physiological situation of embryonic stem cells. In this context, we tested different ECM coatings using control wild type iPSCs in order to understand the conditions that allow the cells to remain in a healthy and undifferentiated state. In addition to this, a critical point is the culturing of iPSCs in xenogenic components of MEFs and Matrigel that may account for the experimental variability among triplicates and this hinder the ability to perform mechanistic studies[26].

In this study, we tested the iPSCs' confluence on xenogeneic-free substrates (i.e., LN-521, Vitronectin, Hu-Fibronectin) using a high-content image analyzer cytometer. The reason for using the automated image-analysis system is due to the fact that counting cells, using the Trypan blue exclusion method would necessitate to make single cell suspensions and this is not recommended when manipulating iPSCs as they should be propagated in cell clusters to avoid cell death. The data obtained with the high-content image-analysis allow us to follow cell confluence without perturbing cells as they are simply imaged every day for 5 days. This technology may be considered as the election method to characterize iPSC lines and it can be included to perform quality control panels of human iPSCs. While we used a commercial software package, the methodology here described can be successfully used by means of equivalent high-content/high-throughput image analysis platforms and similar analytic software packages. The data obtained show that the iPSCs seeded on LN-521 present a linear confluence

during 5 days in culture without splitting the cells and is therefore the best xenogeneic–free substrate tested in this study. One limitation of this protocol is that these results obtained need to be normalized to the first time point in order to consider differences in iPSC attachment to different substrates. Interestingly, the data obtained are most probably driven by an increased cell attachment rate of iPSCs to LN-521. In fact, when normalizing the results for the first time point, no difference is observed among the different substrates.

Based on the results obtained with the study, it would be interesting to better understand the biology of pluripotent stem cells in terms of knowing the major cell surface receptors that mediate cell-ECM contacts and that may be responsible for the maintenance of their self-renewal ability rather than spontaneous differentiation into specific cell types. Interestingly, there are studies showing that the matrix elasticity of the culture surface influenced the differentiation toward different cell types and this is probably dependent on the cell-ECM interactions that activated some intracellular cell-signaling pathway relevant for cell-type specific differentiation[27]. In addition to this, Vigilante et al.[28] explored the genetic contribution to changes in iPSC behavior, by combining computational approaches with gene expression and cell biology datasets. The work by Vigilante et al.[28] is, therefore, a major advance in attempting to map genetic variation to phenotypic variation. These studies may lead to the development of standardized methodologies to be used to perform iPSCs experiments in light of their future possible use in clinics.

ACKNOWLEDGMENTS:

The study was supported by grants from the Fondazione Bambino Gesù and Ricerca Corrente (Italian Ministry of Health) to C.C. We would like to thank Dr Enrico Bertini (Department of Neuroscience, Unit of Neuromuscular and Neurodegenerative Diseases, Laboratory of Molecular Medicine, Bambino Gesù Children's Research Hospital), Rossella Borghi (Genetics and Rare Diseases Research Division, Children's Research Hospital Bambino Gesù, Department of Science, University "Roma Tre"), Giulia Pericoli (Department of Onco-hematology, Gene and Cell Therapy, Children's Research Hospital Bambino Gesù*)* and Roberta Ferretti (Department of Onco-hematology, Gene and Cell Therapy, Children's Research Hospital Bambino Gesù*)* for scientific discussions and technical help. Maria Vinci is recipient of a "Children with Cancer UK fellowship".

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Masotti, A. et al. Aged iPSCs display an uncommon mitochondrial appearance and fail to undergo in vitro neurogenesis. *Aging (Albany NY)*. **6** (12), 1094–1108 (2014).
2. Xu, C. et al. Feeder-free growth of undifferentiated human embryonic stem cells. Nature *Biotechnology*. **19**, 971–974 (2001).
3. Kleinman, H.K. et al. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. *Biochemistry*. **21** (24), 6188-93 (1982).
4. Rodin, S. et al. Clonal culturing of human embryonic stem cells on laminin-521/E-

cadherin matrix in defined and xeno-free environment. *Nature Communications*. **5**, 3195 (2014).
5. Rodin, S., Antonsson, L., Hovatta, O., Tryggvason, K. Monolayer culturing and cloning of human pluripotent stem cells on laminin-521 based matrices under xeno-free and chemically defined conditions. *Nature Prot*ocols. **9** (10), 2354-68 (2014).
6. Laperle, A. et al. α-5 Laminin Synthesized by Human Pluripotent Stem Cells Promotes Self-Renewal. *Stem Cell Reports*. **5** (2), 195-206 (2015).
7. Albalushi, H. et al. Laminin 521 stabilizes the pluripotency expression pattern of human embryonic stem cells initially derived on feeder cells. *Stem Cell International*. **2018**, 7127042 (2018).
8. Zhang, D. et al. Niche-derived laminin-511 promotes midbrain dopaminergic neuron survival and differentiation through YAP. *Science Signaling*. **10** (493) (2017).
9. Lu, H.F. et al. A defined xeno-free and feeder-free culture system for the derivation, expansion and direct differentiation of transgene-free patient-specific induced pluripotent stem cells. *Biomaterials*. **35** (9), 2816-26 (2015).
10. Miyazaki, T., Nakatsuji, N., Suemori, H. Optimization of slow cooling cryopreservation for human pluripotent stem cells. *Genesis*. **52** (1), 49-55 (2014).
11. Bergström, R., Ström, S., Holm, F., Feki, A., Hovatta, O. Xeno-free culture of human pluripotent stem cells. *Methods in Molecular Biology*. **767**, 125-36 (2011).
12. Braam, S.R. et al. Recombinant vitronectin is a functionally defined substrate that supports human embryonic stem cell self-renewal via alphavbeta5 integrin. *Stem Cells*. **26** (9), 2257-65 (2008).
13. Chen, G. et al. Chemically defined conditions for human iPSC derivation and culture. *Nature Methods*. **8** (5), 424-9 (2008).
14. Li, J. et al. Impact of vitronectin concentration and surface properties on the stable propagation of human embryonic stem cells. *Biointerphases*. **5** (3), FA132–42 (2010).
15. Prowse, A.B. et al. Long-term culture of human embryonic stem cells on recombinant vitronectin in ascorbate free media. *Biomaterials*. **31** (32), 8281–8 (2010).
16. Rowland, T.J. et al. Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. *Stem Cells and Development*. **19** (8), 1231–40 (2010).
17. Ruoslahti, E., Engvall, E., Hayman, E.G., Spiro, R.G. Comparative studies on amniotic fluid and plasma fibronectins. *Biochemistry*. **193** (1), 295-9 (1981).
18. Ni, H., Li, A., Simonsen, N., Wilkins, J.A. Integrin activation by dithiothreitol or Mn2+ induces a ligand-occupied conformation and exposure of a novel NH2-terminal regulatory site on the beta1 integrin chain. *Biological Chemistry*. **273** (14), 7981-7 (1998).
19. Seltana, A., Basora, N., Beaulieu, J.F. Intestinal epithelial wound healing assay in an epithelial-mesenchymal co-culture system. *Wound Repair & Regeneration*. **18** (1), 114-22 (2010).
20. Amit, M., Shariki, C., Margulets, V., Itskovitz-Eldor, J. Feeder layer-and serum-free culture of human embryonic stem cells. *Biology of Reproduction*. **70** (3), 837-45 (2004).
21. Vaheri, A., Mosher, D.F. High molecular weight, cell surface-associated glyco-protein (fibronectin) lost in malignant transformation. *Biochimica et Biophysica Acta*. **516** (1), 1-25 (1978).
22. Mao, Y., Schwarzbauer, J.E. Fibronectin fibrillogenesis, a cell-mediated matrix assembly

process. *Matrix Biology*. **24** (6), 389-99 (2005).
23. Polyak, K., Weinberg, R.A. Transitions between epithelial and mesenchymal states: acquisition of malignant and stem cell traits. *Nature Reviews Cancer*. **9** (4), 265-73 (2009).
24. Kadler, K.E., Hill, A., Canty-Laird, E.G. Collagen fibrillogenesis: fibronectin, integrins, and minor collagens as organizers and nucleators. *Current Opinion in Cell Biology*. **20** (5), 495-501 (2008).
25. Hunt, G.C., Schwarzbauer, J.E. Tightening the connections between cadherins and fibronectin matrix. *Developmental Cell*. **16** (3), 327-8 (2009).
26. Villa-Diaz, L.G., Ross, A.M., Lahann, J., Krebsbach, P.H. Concise review: The evolution of human pluripotent stem cell culture: from feeder cells to synthetic coatings. *Stem Cells*. **31** (1), 1-7 (2013).
27. Engler, A.J., Sen, S., Sweeney, H.L., Discher, D.E. Matrix elasticity directs stem cell lineage specification. *Cell*. **126** (4), 677-89 (2006).
28. Vigilante, A. et al. Identifying Extrinsic versus Intrinsic Drivers of Variation in Cell Behavior in Human iPSC Lines from Healthy Donors. *Cell Reports*. **26** (8), 2078-2087.e3 (2019).

Figure 1 Magliocca

Click here to access/download;Figure;Figure 1.jpg

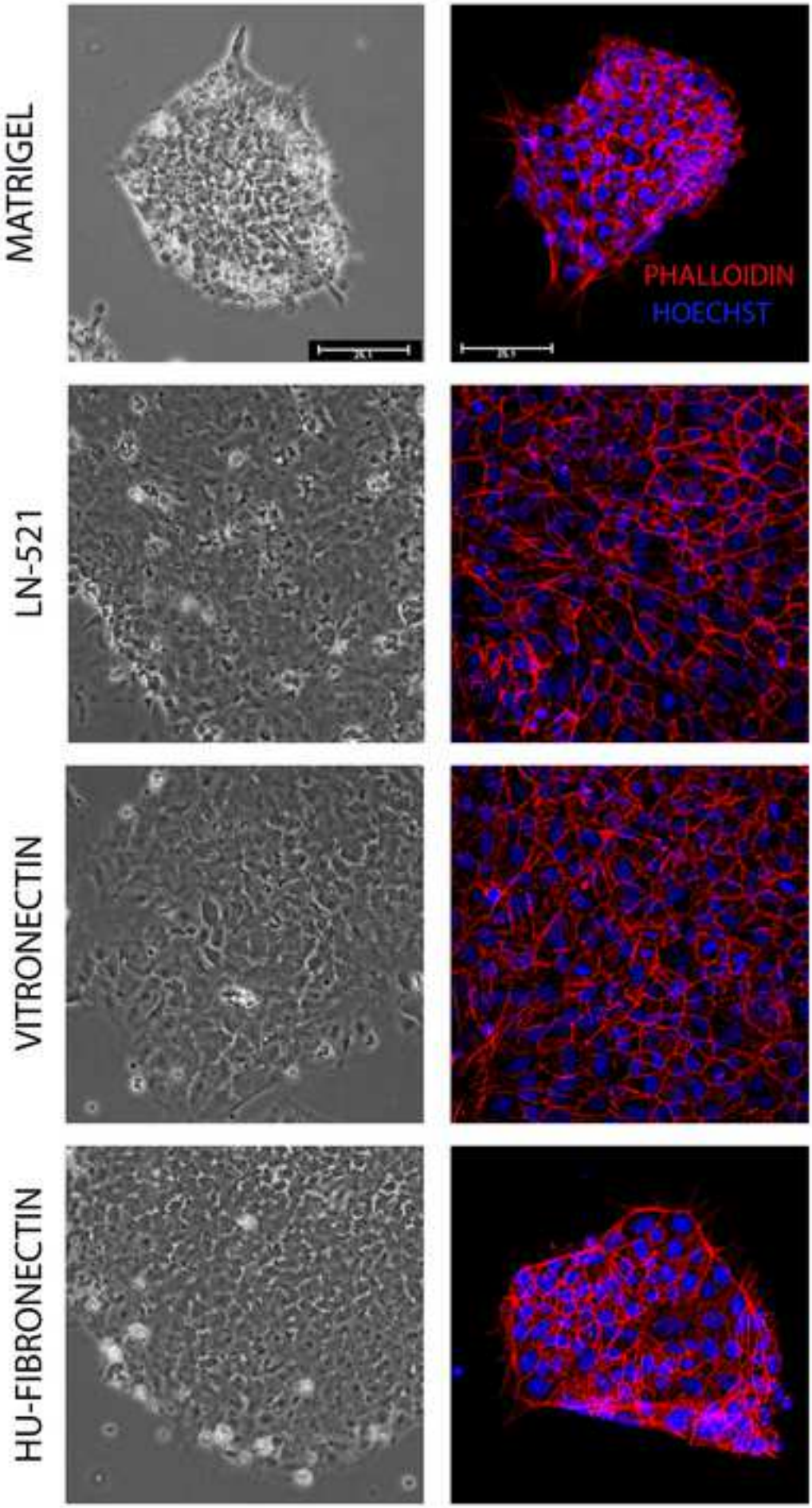


Figure 2 Magliocca

Click here to access/download;Figure;Figure 2.tif

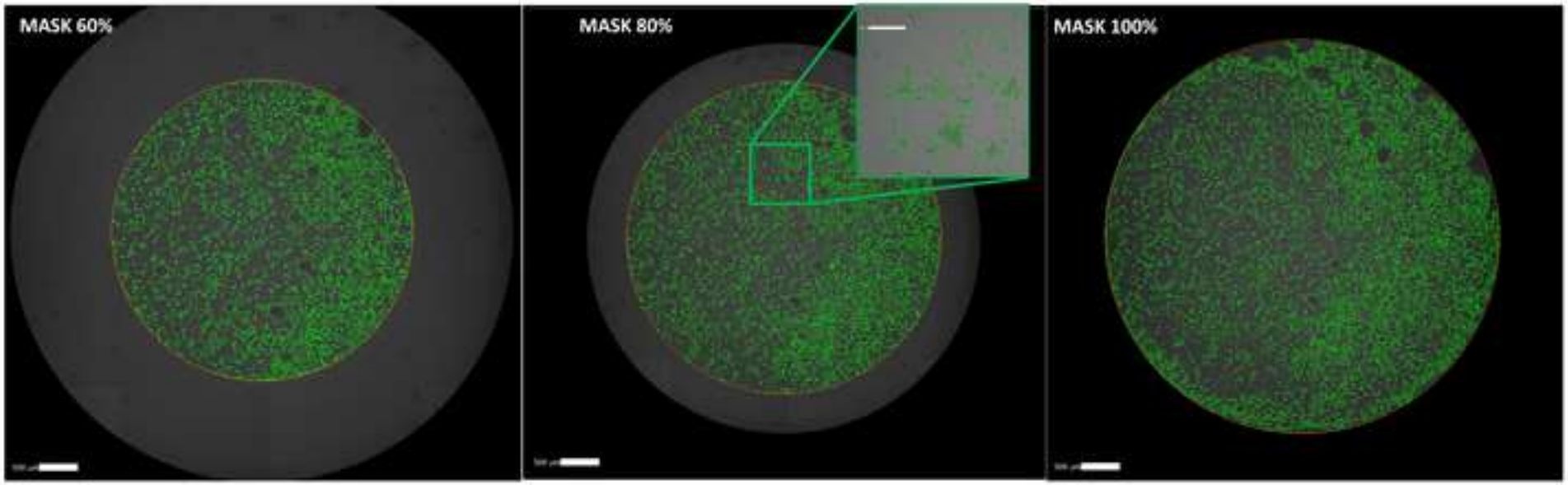


Figure 3 Magliocca

Click here to access/download;Figure;Figure 3.tif

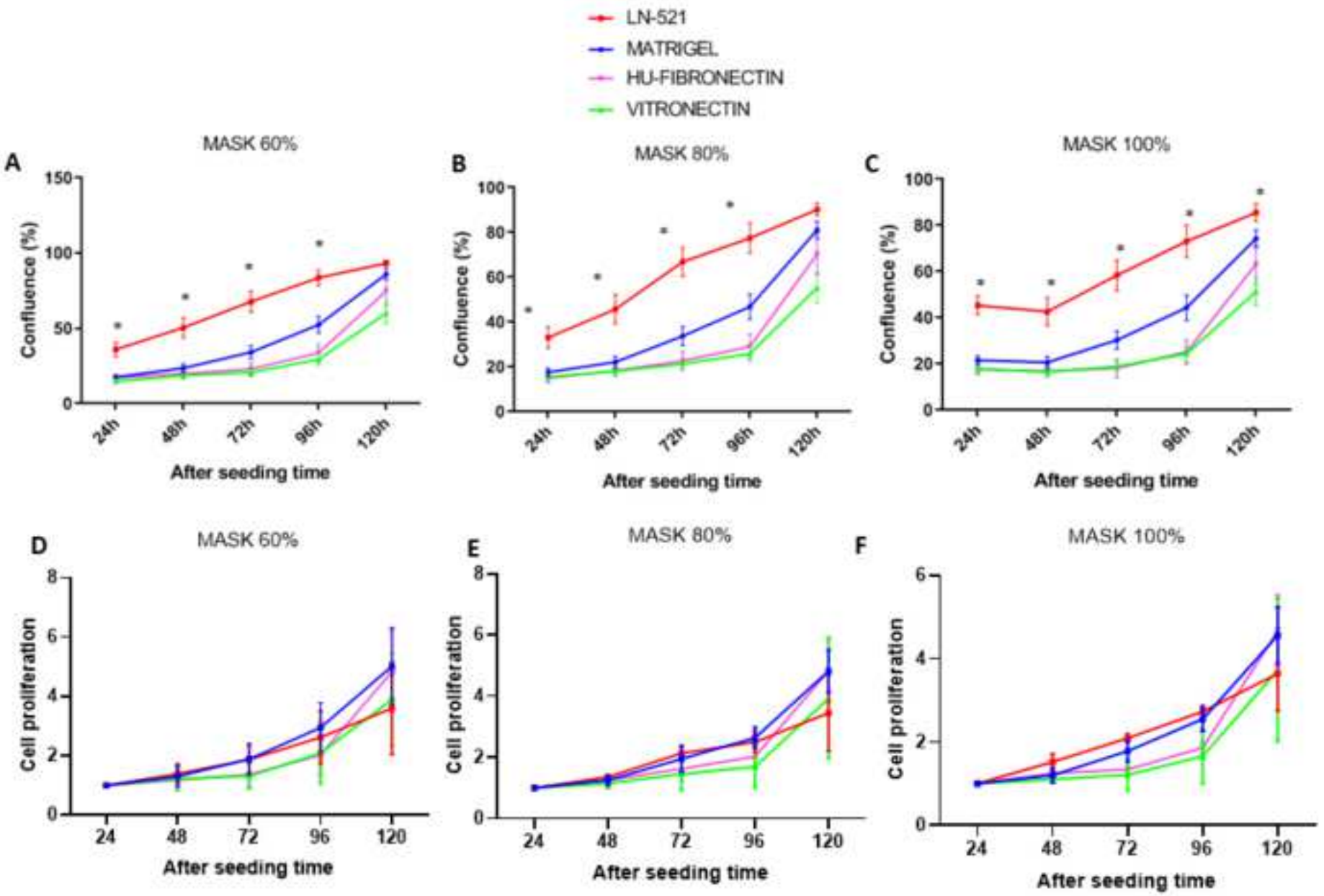


Table 1

Coating compound	Initial Concentration	Final Concentration
HU-Fibronectin	1 mg/mL	10 µg/cm^2
Laminin 521	100 µg/ml	20 µg/mL
Matrigel	*	0.111111111
Vitronectin XF	250 µg/mL	10 µg/mL

Table of Materials

Click here to access/download;Table of Materials;61225_R1 JoVE_Materials alphabetically.xlsx

Name of Material/ Equipment

10 mL Stripette Serological Pipets, Polystyrene, Individually Paper/Plastic Wrapped, Sterile
15 mL high-clarity polypropylene (PP) conical centrifuge tubes
1x PBS (With Ca^{2+}; Mg^{2+})
1x PBS (without Ca^{2+}; Mg^{2+})
5 mL Stripette Serological Pipets, Polystyrene, Individually Paper/Plastic Wrapped, Sterile
Cell culture microplate, 96 WELL, PS, F-Bottom
Cell culture plate, 6 well
DMEM (Dulbecco's Modified Eagle's Medium- high glucose)
EDTA
Epi Episomal iPSC Reprogramming Kit
FAST - READ 102
Fetal Bovine Serum (FBS)
Fibronectin
Glycerol
H_2O
Hoechst
Laminin 521
L-Glutamine (200 mM)
Matrigel
Mouse embryonic fibroblasts (MEF)
MTESR1 Medium
MTESR1 Supplement
Penicillin-Streptomycin (10,000 U/mL)
Phalloidin
Vitronectin
Y-27632

Company	Catalog Number	Comments/Description
Corning	4488	Tool
Falcon	352097	Tool
Thermofisher	14040133	Medium
Euroclone	ECB4004L	Medium
Corning	4487	Tool
Greiner Bio One	655090	Support
Costar	3516	Support
Sigma	D5671	Medium
Sigma	ED4SS-500g	Reagent
Invitrogen	A15960	Reagent
Biosigma	BVS100	Tool
Gibco	10270106	Medium
Merck	FC010	Coating
Sigma	G5516	Reagent
Thermofisher	MILLIQ 33342	Reagent
Stem Cell Technologies	77003	Coating
Gibco	LS25030081	Reagent
Corning Matrigel hESC-Qualified Matrix	354277	Coating
Life Technologies	A24903	Coating
Stem Cell Technologies	85851	Medium
Stem Cell Technologies	85852	Medium
Gibco	15140122	Reagent
Sigma	P1951	Reagent
Stem Cell Technologies	7180	Coating
Sigma	Y0503	Reagent

Bambino Gesù
OSPEDALE PEDIATRICO

Roma, 21/03/2020

Dear Prof. Nam Nguyen,

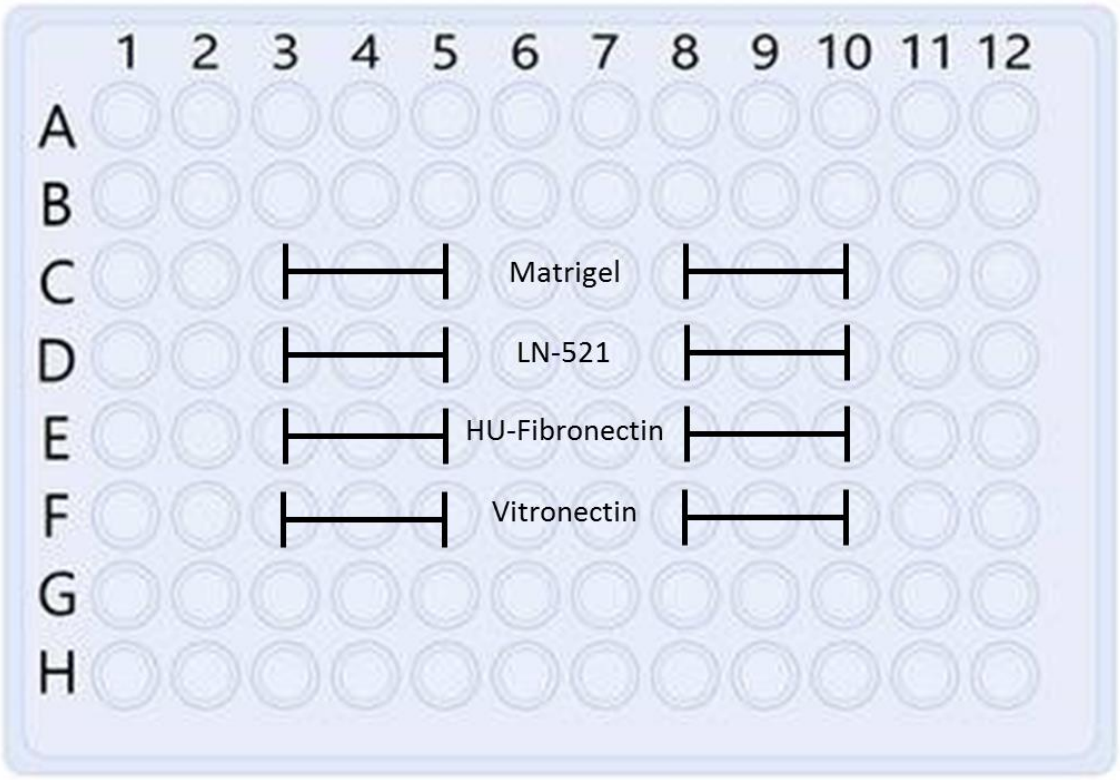
I hope that this finds you well. I wish to thank you for your consideration of the manuscript (titled: "*Measuring the confluence of iPSCs using an automated imaging system*" by Valentina Magliocca, Maria Vinci, Tiziana Persichini, Franco Locatelli, Marco Tartaglia, Claudia Compagnucci) that we submitted to Jove, We have respectfully amended the manuscript following your comments.

With best regards,
Claudia Compagnucci

Claudia Compagnucci, PhD
Genetics and Rare Diseases Research Division
Ospedale Pediatrico Bambino Gesù
Viale di San Paolo, 15
00146 Rome, Italy

Supplemental file

Picture explaining how the different coating have been disposed in the 96-well plate.



Experimental parameters:

To Acquire the images:

- Application: *Confluence*
- Image Acquisition setting: *Auto Exposure*
- Configuration: *Brightfield*
- Acquisition resolution: *1um/pixel*
- For the *Contrast: auto contrast*
- For the Focus set up:
- *"hardware autofocus – register auto mode"*
- *Target focal plane bright focus*.

For the Analysis:

- Application: *Confluence*
- Analysis resolution*: 2um/pixel*
- Algorithm*: Texture*
- Intensity threshold: *15*
- Precision*: High*
- Min cluster size*: 50 µm^3*

Imaging parameters used

Channel Name	Name	Cube	Contrast Method	Emission Wavelength	Peak Emission	Peak Excitation
Channel 1	DAPI	DAPI	FLUO	nm	461	0 nm
Channel 2	RHOAD	RHOAD	FLUO	nm	578	0 nm

Channel Name	Lookup table name	Exposure time	Gain	Differential interference contrast	Condenser	Resolution XY	Resolution Z
Channel 1	Blue	220.02 ms	0	-		0.469 µm	1.281 µm
Channel 2	Red	795 ms	0	-		0.469 µm	1.281 µm

Camera settings	
Camera	Hamamatsu-C11440-42U-USB-001428
Format	--
Digitization	16 bits
Gamma	0.9807789
Quality Mode	1 MHz
Image Flip X Axis	No
Image Flip Y Axis	No
Black-Value	0
White-Value	65535
Shading Correction	OFF

Microscope Settings	
System Name	**LAS X**
Microscope Model	DMI8
OBjective	HC PL FLUOTAR L 40x/0.60 DRY
Order number (Obj.)	11506201
Numerical aperture	0.6
Refractive index	1
Mounting Medium Refraction index	
Z Movement	Z then Lambda
Camera Light	100%