Journal of Visualized Experiments

Ordering single cells and single embryos in 3D confinement: a new device for high content screening

--Manuscript Draft--

Manuscript Number:	JoVE51880R3
Full Title:	Ordering single cells and single embryos in 3D confinement: a new device for high content screening
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Microcavities; Replica Molding; Microfabrication; High-content-screening; 3-Dimensions; Single Cells; Nucleus; Golgi; Cytokinetic Ring.
Manuscript Classifications:	10.1.293.69: Bioengineering; 14.6.850.520.308: Data Collection; 14.6.850.520.308.250: Health Surveys; 5.1.370: Diagnostic Techniques and Procedures; 5.1.370.500: Mass Screening; 5.5.916: Technology, Pharmaceutical; 5.5.916.680: High-Throughput Screening Assays; 7.4.299: Cell Physiological Processes; 7.4.299.134.220: Cell Division; 7.4.366: Cellular Microenvironment
Corresponding Author:	Daniel Riveline, Ph.D. Lab Cell Physics ISIS/IGBMC Strasbourg, Cedex FRANCE
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	riveline@unistra.fr
Corresponding Author's Institution:	Lab Cell Physics ISIS/IGBMC
Corresponding Author's Secondary Institution:	
First Author:	Viktoria Wollrab
First Author Secondary Information:	
Other Authors:	Viktoria Wollrab
	David Caballero
	Raghavan Thiagarajan
Order of Authors Secondary Information:	
Abstract:	Biological cells are usually observed on flat (2D) surfaces. This condition is not physiological, and phenotypes and shapes are highly variable. Screening based on cells in such environments have therefore serious limitations: cell organelles show extreme phenotypes, cell morphologies and sizes are heterogeneous and/or specific cell organelles cannot be properly visualized. In addition, cells in vivo are located in a 3D environment; in this situation, cells show different phenotypes mainly because of their interaction with the surrounding extracellular matrix of the tissue. In order to standardize and generate order of single cells in a physiologically-relevant 3D environment for cell-based assays, we report here the microfabrication and applications of a device for in vitro 3D cell culture. This device consists of a 2D array of microcavities (typically 105 cavities/cm2), each filled with single cells or embryos. Cell position, shape, polarity and internal cell organization become then normalized showing a 3D architecture. We used replica molding to pattern an array of microcavities, 'eggcups', onto a thin polydimethylsiloxane (PDMS) layer adhered on a coverslip. Cavities were covered with fibronectin to facilitate adhesion. Cells were inserted by centrifugation. Filling percentage was optimized for each system allowing up to 80%. Cells and embryos viability was confirmed. We applied this methodology for the visualization of cellular organelles, such as nucleus and Golgi apparatus, and to study active processes, such as the closure of the cytokinetic ring during cell mitosis. This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure and

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

	compacted phenotypes for Golgi and nucleus alignment. We characterized the method for mammalian cells, fission yeast, budding yeast, C. elegans with specific adaptation in each case. Finally, the characteristics of this device make it particularly interesting for drug screening assays and personalized medicine.
Author Comments:	This submission is an invited contribution proposed by Rachel Baker and Elizabeth Sheeley. We agreed that, if the manuscript is accepted, we will keep control about the moment it is published because other works currently under preparation should be accepted first.
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	
If this article needs to be filmed by a certain date to due to author/equipment/lab availability, please indicate the date below and explain in your cover letter.	

Powered by Editorial Manager® and ProduXion Manager® from Aries Systems Corporation

Strasbourg, December 17th 2013

Dear Editor,

We are submitting a manuscript entitled ´**Ordering cells and embryos in 3 dimensions: a new device for high content screening**´.

Standard cell-based assays are usually performed on flat surfaces, where cells spread and take quasi-2D shapes. In addition, and amongst other limitations, cells are disordered, show heterogeneous phenotypes and some organelles are not properly visualized. This has important consequences, in particular, for screening applications. In contrast, cells *in vivo* are located in a three dimensional environment showing phenotypes and activities very different from those observed on standard ´flat´ assays. This demands new devices and methods to bridge the gap between *in vitro* and *in vivo* conditions with reproducible and standardized conditions.

In this paper, we report the microfabrication and application of a new *in vitro* three dimensional and high-content-screening device for cell-based applications. This method allows the study of cell architecture as well as dynamical processes with high-content-screening characteristics. Our laboratory focuses in the biophysical description of cellular phenomena, such as cytoskeleton organization, cell division and drug screening. Our developed 3D cell culture device and method allow to standardize and to generate order of individual cells in a 3D environment. This device consists of an array of thousands to billions of microcavities, each filled with individual cells, where cell position, shape, and internal cell organization become normalized with a 3D architecture. We report that cells in this configuration showed phenotypes similar to those observed *in vivo*, and most importantly, we identified new dynamic processes that could not be observed using standard assays.

This method is simple, fast and flexible. The device can be easily adapted for different cell types and model systems and can be used for a variety of applications if the procedure is clearly described and characterized. We believe that this new device and method will be useful to the scientific community and this motivated the submission of our work in JoVE's special multimedia format.

This submission is an invited contribution proposed by Rachel Baker and Elizabeth Sheeley. We agreed that, if the manuscript is accepted, we will keep control about the moment it is published because other works currently under preparation should be accepted first. This paper has not been published or is under consideration in another journal. We would like you to consider this manuscript for publication as an article in *JoVE* journal.

Sincerely yours,

Daniel Riveline.

Author contributions:

D.R. initiated the device and method for fission yeast. V.W. and D.R. designed the approach for mammalian cells. V.W. and D.C. performed the experiments with mammalian cells and *C. elegans*. D.R., R.T. and V.W., performed the experiments with yeast. All the authors analyzed the data and wrote the paper. D.R. supervised the project.

For correspondence:

Daniel Riveline
E-mail: riveline@unistra.fr

Laboratory of Cell Physics ISIS/IGBMC
Institut de Science et d'Ingénierie Supramoléculaires (ISIS)
CNRS UMR 7006 et Université de Strasbourg
8 allée Gaspard Monge - BP 70028
67083 Strasbourg Cedex,France
Tel: 33 (0)3 68 85 51 64
Fax: 33 (0)3 68 85 52 32

Suggested Reviewers:

1- *Dr. Elena Martínez*
Biomimetic systems for cell engineering-IBEC (Institute for Bioengineering of Catalonia)
Barcelona, Spain
E-mail: emartinez@ibecbarcelona.eu

2- *Dr. Christopher A. Mills*
Nanoelectronics Research Centre at the Advanced Technology Institute, Surrey University
Surrey, UK
E-mail: c.mills@surrey.ac.uk

3- *Dr. François Bessueille*
Institute de Sciences Analytiques – Laboratoire des Sciences Analytiques, Université Claude Bernard-Lyon1
Lyon, France
E-mail: francois.bessueille@univ-lyon1.fr

4- *Dr. Jose-Antonio Plaza*
Centre Nacional de Microelectronica CNM-IMB, CSIC
Bellaterra, Spain
E-mail : joseantonio.plaza@imb-cnm.csic.es

5- *Dr. Javier Gomez Fernandez*
Wyss Institute for Biologically Inspired Engineering, University of Harvard
Cambridge, US
E-mail : Javier.fernandez@wyss.harvard.edu

6- *Dr. Ana Ruiz*
Department of Applied Biotechnology and Translational Medicine, University of Milan
Milan, Italy
E-mail : ana.ruiz@unimi.it

Excluded Reviewers:

1- *Dr. Fred Chang*
University of Columbia
New York, US
E-mail: fc99@columbia.edu
Reason: Direct competitor.

2- *Dr. Mohan Balasubramanian*
Mechanobiology Institute, NUS
Singapore
E-mail: mohan@tll.org.sg
Reason: Direct competitor.

3- *Dr. Phong Tran*
Institut Curie
Paris, France
E-mail: phong.tran@curie.fr
Reason: Direct competitor.

Manuscript
Click here to download Manuscript: 51880_R1_RE.docx

TITLE: Ordering single cells and single embryos in 3D confinement: a new device for high content screening

Authors:
Wollrab, Viktoria[1,2]
[1]Laboratory of Cell Physics
Institut de Science et d'Ingénierie Supramoléculaires (ISIS), CNRS and Université de Strasbourg.
[2]Development and Stem Cells Program
Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS and Université de Strasbourg.
Strasbourg, France
wollrab@unistra.fr

Caballero, David[1,2]
[1]Laboratory of Cell Physics
Institut de Science et d'Ingénierie Supramoléculaires (ISIS), CNRS and Université de Strasbourg.
[2]Development and Stem Cells Program
Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS and Université de Strasbourg.
Strasbourg, France
caballero@unistra.fr

Thiagarajan, Raghavan[1,2]
[1]Laboratory of Cell Physics
Institut de Science et d'Ingénierie Supramoléculaires (ISIS), CNRS and Université de Strasbourg.
[2]Development and Stem Cells Program
Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS and Université de Strasbourg.
Strasbourg, France
thiagara@igbmc.fr

Riveline, Daniel[1,2]
[1]Laboratory of Cell Physics
Institut de Science et d'Ingénierie Supramoléculaires (ISIS), CNRS and Université de Strasbourg.
[2]Development and Stem Cells Program
Institut de Génétique et de Biologie Moléculaire et Cellulaire (IGBMC), CNRS and Université de Strasbourg.
Strasbourg, France
riveline@unistra.fr

Corresponding author: Daniel Riveline; Laboratory of Cell Physics ISIS/IGBMC, Université de Strasbourg and CNRS (UMR 7006), 8 allée Gaspard Monge, Strasbourg, 67083 France; riveline@unistra.fr TEL: +33 (0) 368 855 164.

Keywords: Microcavities, Replica Molding, Microfabrication, High-content-screening, 3-Dimensions, Single Cells, Nucleus, Golgi, Cytokinetic Ring.

Short Abstract: We report a device and a new method to study cells and embryos. Single cells are precisely ordered in microcavity arrays. Their 3D confinement is a step towards 3D environments encountered in physiological conditions and allows organelle orientation. By controlling cell shape, this setup minimizes variability reported in standard assays.

Abstract: Biological cells are usually observed on flat (2D) surfaces. This condition is not physiological, and phenotypes and shapes are highly variable. Screening based on cells in such environments have therefore serious limitations: cell organelles show extreme phenotypes, cell morphologies and sizes are heterogeneous and/or specific cell organelles cannot be properly visualized. In addition, cells *in vivo* are located in a 3D environment; in this situation, cells show different phenotypes mainly because of their interaction with the surrounding extracellular matrix of the tissue. In order to standardize and generate order of single cells in a physiologically-relevant 3D environment for cell-based assays, we report here the microfabrication and applications of a device for *in vitro* 3D cell culture. This device consists of a 2D array of microcavities (typically 10^5 cavities/cm^2), each filled with single cells or embryos. Cell position, shape, polarity and internal cell organization become then normalized showing a 3D architecture. We used replica molding to pattern an array of microcavities, 'eggcups', onto a thin polydimethylsiloxane (PDMS) layer adhered on a coverslip. Cavities were covered with fibronectin to facilitate adhesion. Cells were inserted by centrifugation. Filling percentage was optimized for each system allowing up to 80%. Cells and embryos viability was confirmed. We applied this methodology for the visualization of cellular organelles, such as nucleus and Golgi apparatus, and to study active processes, such as the closure of the cytokinetic ring during cell mitosis. This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure and compacted phenotypes for Golgi and nucleus alignment. We characterized the method for mammalian cells, fission yeast, budding yeast, *C. elegans* with specific adaptation in each case. Finally, the characteristics of this device make it particularly interesting for drug screening assays and personalized medicine.

Introduction:

Current *in vitro* cell-based assays are two-dimensional (2D). This configuration is not natural for mammalian cells and therefore is not physiologically relevant [1]; cells show a diversity of shapes, sizes and heterogeneous phenotypes. They present additional serious limitations when applied to screening applications, such as a disordered distribution within the plane and extreme phenotypes of cellular organelles (stress fibers, in particular). This is particularly important in clinical trials for drug testing, where high budgets are spent each year. Most of these drugs though fail when applied to animal models because of the artificial 2D culture condition in early stages of drug screening. In addition, by using this approach, specific cell organelles cannot be properly visualized, such as the cytokinetic actomyosin ring during cell mitosis, and generally structures that are evolving in the plane perpendicular to the plane of observation. Some new 2D assays have been proposed in order to overcome the above-mentioned drawbacks and

important insights on cytoskeleton organization have been observed [2,3]. However, these assays still present one serious limitation: cells show a very spread phenotype in contrast to what is observed *in vivo*, where cells present a 3D architecture. These artifacts associated with the culture method may trigger non-physiological features such as enhanced stress fibers [1,4,5].

Three-dimensional cell culture assays provide multiple advantages when compared to 2D environments [6,7]. They are physiologically more relevant, and results are therefore meaningful. As an example, cells embedded in hydrogels show 3D-like structures but their morphologies differ from one cell to another [8,9]; this is critically important for screening applications. An alternative strategy is to embed single cells in microfabricated cavities [10,11]. Cell position, shape, polarity and internal cell organization can then become normalized. Besides providing 3D-like architecture to cells, microcavities also allows for high-content screening studies [10,12-14]; single cells can be ordered into microarrays and cellular organelles and their evolutions can be observed in parallel. This regularity provides good statistics with low number of cells and better temporal/spatial resolutions. Hits are easier to identify reliably.

In this study, we show the fabrication and application of a new 3D-like single cells culture system for high-content-screening applications [10,12,13]. The device consists of an array of elastomeric microcavities (10^5 cavities/cm^2), coined 'eggcups' (EC). Dimensions and total volume of EC in this work are optimized to the typical volume of individual NIH3T3 and HeLa cells during cell division in 2D. Morphology of the cavities – cylindrical – is selected to properly orient cell shape for the visualization of active processes. Replica molding is used to pattern an array of EC onto a thin polydimethylsiloxane (PDMS) layer adhered on a glass coverslip [15,16]. Cells are introduced in the EC by centrifugation. We report here observation and normalization of cellular organelles (actin stress fibers, Golgi apparatus and nucleus) in 3D (EC) in comparison with the same cells on 2D (flat) surfaces. We also report the observation of active dynamical processes such as the closure of the cytokinetic actomyosin ring during cell mitosis. Finally, we show results of this methodology on other systems with rigid walls, such as budding yeast, fission yeast and *C. elegans* embryos which confirms the applicability of our methodology to a wide range of model systems.

We next present a detailed and exhaustive protocol in order to fabricate and apply the 'eggcups' for 3D microfabrication. Our approach is simple and does not need a clean room. We anticipate that this new methodology will be particularly interesting for drug screening assays and personalized medicine, in replacement of the Petri dishes. Finally, our device will be useful for studying the distributions of cells responses to external stimuli, for example in cancer [17] or in basic research [18].

Protocol:

1. Microfabrication of 'eggcups'

1.1 Fabrication of the master: microcavities array

1.1.1 Heat a 3'' silicon wafer up to 200 °C to evaporate any presence of humidity.

1.1.2 Spin-coat a thin layer of SU-8 photoresist. Adjust the volume of resin and spinning speed depending on the desired thickness and photoresist type. This thickness will dictate the depth of the EC. For a 30 µm thick layer and SU-8 2025, spin-coat at 2800 rpm.

1.1.3 Pre-bake the wafer at 65 °C for 1 min (step 1 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details.

1.1.4 Pre-bake the wafer at 95 °C for 3 min (step 2 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details.

1.1.5 Load the wafer on the mask aligner for UV exposure. Place the photolithography mask on it. The mask shows a pattern of circular features (disks) of 20 µm in diameter. . Ensure a perfect contact between each other.

NOTE: Different manufacturers offer photolithography masks. The spatial resolution will determine the final cost. Acetate masks provide acceptable resolution (~10 µm) at low cost. Chromium masks provide better resolution but are more expensive. Adapt the diameters of disks (from the photolithography mask) to the volume of cells. Dimensions of disks on the mask will determine the diameter of cavities in the device. Small diameters will lead to a low filling; too large diameter will not confine the cells. For HeLa and NIH3T3 cells, diameters of 20 µm to 25 µm are suggested.

1.1.6 Check the power of the UV lamp prior exposition and optimize the exposure time accordingly. Irradiate (wavelength=365 nm) for 41.5 s (or the optimized exposure time) at 250 mJ/cm^2.

NOTE: SU-8 2025 is a negative photoresist, which means that exposed regions to UV will be cured. In this case, the circular features were black and the rest transparent. Positive photoresist work in the opposite way: non-exposed regions are cured. Select the photoresist accordingly, depending on the design and photo-mask.

Protect the eyes from UV light with appropriate safety glasses.

1.1.7 Remove gently the mask from the photoresist layer.

1.1.8 Post-bake the wafer at 65 °C for 1 min (step 1 of 2) for a 30 µm thick SU-8 2025 layer. Post-bake the wafer at 95 °C for 3 min (step 2 of 2) for a 30 µm thick SU-8 2025 layer. Adapt the time depending on the photoresist type and thickness desired. Check the manufacturer datasheet for details. After the post-baking, cool the wafer to room temperature on the bench for around 1 min.

1.1.9 Place the wafer in the spin-coater and drop few milliliters of SU-8 developer to cover the whole wafer area. Develop for 2 min and then spin-coat at 1000 rpm for 30 s. Repeat the procedure three times.

1.1.10 Rinse with 2-propanol to ensure the complete removal of undeveloped SU-8. Appearance of white regions is an indication of incomplete development. If so, repeat the developing step an additional time.

1.1.11 Hard-bake the wafer at 200 °C to ensure robustness of the fabricated microstructures. This step is optional.

1.1.12 Store the 3'' wafer with microstructures inside a 94 mm x 15 mm polystyrene Petri dish.

NOTE: There is no need for surface treatment, in particular silanization, for the next steps.

1.2 Fabrication of the polydimethylsiloxane (PDMS) replica: pillars array

1.2.1 Thoroughly mix in a 1:10 ratio (w/w) the cross-linker and the pre-polymer for a total of 30 g inside a 50 ml tube.

NOTE: Using a 1:10 (v/v) ratio is also working.

1.2.2 Centrifuge the tube at 1800 x *g* for 5 min to remove air bubbles.

1.2.3 Drop gently the PDMS on top of the microstructures.

NOTE: If air bubbles appear during this step, degas the sample using a vacuum pump for 15-20 min.

1.2.5 Place the sample in an oven at 65 °C for 4 h.

NOTE: The curing time varies between users and, together with the cross-linker:pre-polymer proportion. This time will dictate the rigidity of the PDMS. It is recommended to cure more than 2 h and to stick to a fixed curing time.

1.2.6 Use a scalpel to gently cut the area of interest (stamp) of about 1 cm^2 which includes around 10^5 microcavities or 'eggcups'.

NOTE: Cut first the PDMS and then, peel it off gently. Check the quality of the PDMS replica with an optical microscope.

1.3 Fabrication of 'eggcups' by replica molding

In the following, two alternative strategies for the fabrication of 'eggcups' are described. Both protocols are similar and provide identical results:

1.3.1 Strategy 1

1.3.1.1 Activate the fabricated PDMS stamp by oxygen plasma treatment for 30 s. Store temporarily the activated stamps into a closed Petri dish to prevent deposition of dust.

NOTE: Adjust the exposition time if other gases for the plasma are used.

1.3.1.2 Place the activated PDMS stamp upside up (the side with the structures) in a Petri dish next to a 15 ml cap. Fill the cap with 200 µl of Trimethylchlorosilane (TMCS). Close the Petri dish and let the stamp silanize for 7 min.

NOTE: Some temporary deformation on the stamp and/or change in color (white) may be observed. The stamp will recover its original shape in short time and the structures will not be affected.

CAUTION: TMCS produces acute inhalation and dermal toxicity, and is highly flammable (with ignition flashback able to occur across considerable distances). Consequently, it should be used in a fume cupboard away from sources of ignition.

1.3.1.3 Place the PDMS stamp on the spin-coater with the structures upside up. Put a small drop of few microliters (around 20 µl) of liquid PDMS (1:10 w/w cross-linker:pre-polymer) on top of the structures. Spin-coat at 1500 rpm for 30 s to deposit a thin layer of PDMS on top of the structures.

NOTE: If the stamp does not fit the spin-coater chuck place the stamp on top of a Petri dish lid with a small hole at its center.

1.3.1.4 Place the stamp in the oven at 65 °C for 4 h to cure the deposited spin-coated PDMS layer.

1.3.1.5 Activate the thin PDMS layer by placing the PDMS stamp upside up, together with a glass coverslip #0 of 25 mm in diameter, using oxygen plasma cleaner for 30 s. Proceed quickly to the next step.

NOTE: Coverslips with other thicknesses, shapes, and dimensions can be used as well. However, some cellular structures could be difficult to visualize depending on the selected coverslip thickness and objective magnification and/or NA and/or working distance. Check the objective data sheet.

1.3.1.6 Place in contact the stamp (the side with the thin spin-coated layer) with the glass coverslip. Press gently all around the surface of the stamp with tweezers to make the 'bonding'. Finally, keep a constant pressure on top of the stamp for around 10 s.

1.3.1.7 After 30 min gently 'peel' the stamp out of the coverslip in order to 'liberate' 'eggcups' (see Figure 1). Rinse thoroughly with ethanol and dry. If PDMS 'eggcups' are not well adhered on the glass coverslip (i.e. they detach during the 'unpeeling' step), consider adjusting the settings of the plasma cleaner and restart at step 1.3.1.5.

CAUTION: This step is delicate. Pay attention in order to avoid breakage of the coverslip and/or detachment of the thin PDMS layer.

1.3.1.8 Glue a small piece (handle) of cured PDMS of 1 mm x 1 mm x 3 mm in volume at the edge of the coverslip with a small drop of liquid PDMS and cure the PDMS as before. This will facilitate the manipulation of the sample afterwards (see Figure 1). This step is optional.

1.3.2 Strategy 2

1.3.2.1 Hydrophilize the fabricated PDMS stamps by oxygen plasma treatment for 30 s. Store temporarily the activated stamps in a closed Petri dish to prevent deposition of dust.

NOTE: Adjust the exposition time if other gases for the plasma are used.

1.3.2.2 Hydrophilize the 25 mm diameter glass coverslips #0 oxygen plasma treatment at 15 W for 30 s. Proceed quickly to the next step.

NOTE: Coverslips with other thicknesses, shapes, and dimensions can be used as well. However, cellular structures will be difficult to visualize depending on the selected coverslip thickness and objective characteristics (see note above).

1.3.2.3 Spin-coat a small drop of PDMS (1:10 w/w cross-linker:pre-polymer) of few microliters onto the glass coverslips. Spin-coat at 1500 rpm for 30 s for a final thickness of around 50 µm.

1.3.2.4 Glue a small piece (handle) of cured PDMS of 1 mm x 1 mm x 3 mm in volume at the edge of the coverslip with a small drop of liquid PDMS and cure the PDMS as before. This will facilitate the manipulation of the sample afterwards (see Figure 1). This step is optional.

1.3.2.5 Store temporarily the PDMS-coated coverslips onto a clean wipe inside a Petri dish to protect from dust deposition.

1.3.2.6 Put a drop of siliconizing reagent on top of each stamp and let it evaporate for 1-2 min. Then, dry them under a stream of N_2.

NOTE: In this step, a temporary deformation of the stamp can be observed during evaporation. The stamp will recover its original shape after drying with N_2 without any permanent deformation of microstructures.

1.3.2.7 Drop very gently the silanized stamp on top of the PDMS-spin-coated glass coverslip stored in the Petri dish. Make sure that both sides are completely parallel during the contact. Avoid pressing or moving the stamp after placing it onto the PDMS-coated coverslip.

1.3.2.8 Place the Petri dish with samples in the vacuum for 1-2 h to remove air bubbles.

CAUTION: Ensure that samples are totally horizontal to avoid stamp displacement. Avoid also vibration potentially caused by the vacuum pump.

1.3. 2.9 Place the samples in the oven at 65 °C for 4 h.

1.3.2.10 Gently, peel off the stamp to reveal ‘eggcups’. Rinse thoroughly with ethanol and dry.

NOTE: Practice at this point is needed. Pay attention in avoiding breakage of the coverslip and/or detachment of the thin PDMS layer.

2. Introducing cells into the 'eggcups'

In order to introduce mammalian cells inside 'eggcups', PDMS surface needs to be functionalized with adhesion proteins of the extracellular matrix. This example uses fibronectin but other proteins of interest, such as collagen, could be used.

2.1 Hydrophylize the 'eggcups' in the oxygen plasma cleaner for 30 s.

NOTE: Optimize the parameters if needed.

2.2 Prepare a solution in PBS 1X of 20 µg ml^{-1} fibronectin from Bovine sources.

2.3 Sterilize the EC with UV for 5 min. Deposit a small drop (around 20-50 µl) of fibronectin solution to cover the entire EC area and incubate for 1h at RT. Protect the sample from drying.
2.4 Rinse gently the 'eggcups' with PBS 1X. Repeat 3 times.

NOTE: The sample is ready to use immediately or stored at 4 °C in the dark for several weeks.

2.5 Introduce a cylindrical custom-made plastic piece of 63 mm in height, 26 mm of external radius and 7 mm of internal radius dimensions into a 50 ml tube (see Figure 2) [19]

CAUTION: Use UV-sterilized items or sterilize them prior use.

NOTE: This piece can be easily fabricated in the lab. or by any available machine shop.

2.6 Put 13 ml of cell culture medium inside the tube (see Table 1). The medium should fill at least 2 cm above the cylindrical piece to ensure complete immersion of the sample.

NOTE: For details of specific cell types, and other model systems such as yeast or *C. elegans* embryos, and the corresponding medium used, refer to section 5 and Table 1. The described protocol was optimized for HeLa, NIH3T3 cells, and other cell lines (see Table).

2.7 Introduce very gently the 'eggcups' inside the tube and parallel to the upper side of the plastic piece. Use sharp tweezers to hold the sample using the PDMS handle. Press gently the coverslip until it lies on top of the upper side of the plastic piece, until it is fully immersed (see Figure 2).

NOTE: It is recommended to use sharp and straight tweezers. With curved tweezers, the manipulation of the sample is difficult and may cause breakage.

2.8 Culture cells until 80 % - 100 % confluence in a P60 Petri dish and collect them by trypsinization.

NOTE: Cells can be wild-type, transfected or treated with any drug of interest.

CAUTION: Avoid the formation of cell aggregates which will avoid single cells to penetrate inside 'eggcups'. To optimize this step, pipette up and down thoroughly after trypsinization.

2.9 Re-suspend cells into 5 ml culture medium. Pipette 200 µl of cells on top of the 'eggcups'.

CAUTION: Drop cells as centered as possible on top of the 'eggcups' but avoiding physical contact. This will prevent breakage and/or damage of the sample.

2.10 Centrifuge at 1800 x *g* for 2 min.

NOTE: After the first centrifugation, check in a microscope the filling percentage of the 'eggcups'.

2.11 Pipette again 200 µl of cells on top of the 'eggcups' and centrifuge at 1800 x *g* for 2 min. Repeat for a total of three times in order to optimize the filling percentage.

NOTE: After the last centrifugation, check with a microscope the filling percentage of 'eggcups'. If necessary, repeat the filling + centrifugation steps until reaching the desired filling percentage.

2.12 Remove the sample from the tube using the sharp tweezers holding the PDMS handle. Make sure to be careful in not 'disturbing' cells which are held in the 'eggcups' (see Figure 2).

2.13 Place the sample in a Petri dish with medium. Rinse to remove the excess of cells which are not in the 'eggcups' by pipetting up and down three times gently next to each side (total 4 sides) of the microstructure array.

CAUTION: Pipetting too strongly may release some cells out from the 'eggcups'.

2.14 Replace the medium with fresh medium to remove non attached cells.

NOTE: In this step a drug of interest can be added.

2.15 Fix cells or prepare them for time-lapse imaging. See step 4.1.

3. Observation of active cellular dynamics in 'eggcups': cytokinetic ring closure

This example uses HeLa cells which are transfected with MYH10-GFP and Lifeact-mcherry for myosin and actin, respectively, key active molecules involved in the cytokinetic ring closure during cell mitosis. The device is prepared with microcavities of 25 µm in diameter. For their observation, an epifluorescence inverted microscope was used, equipped with a 60X oil objective (1.40 NA, DIC, Plan Apo) and GFP (myosin) and TxRed (actin) filters. Alternatively an upright confocal microscope was used, equipped with a 25X or 63X HCX IR APO L water objective (0.95 NA). For this example, it is highly recommended to synchronize cells by using the double thymidine block, mitotic block or mitotic shake-off method [20-23].

NOTE: The thickness of the PDMS used for the 'eggcups' allows the usage of a variety of objectives both in inverted and upright positioned microscopes.

3.1 Place ‘eggcups’ into a microscope holder and fill it with 1 ml of 10 % FCS L-15 observation medium. To avoid evaporation, place a glass lid on top of the holder or apply a thin layer of mineral oil on top of the medium. Select the 60X oil objective.

NOTE: L-15 medium is adequate for non CO_2 atmospheres. Note also that some compounds of DMEM are auto-fluorescent. When using this medium, it is recommended to photobleach the fluorescent compounds by illuminating it with a high intensity lamp for 1-2 h.

NOTE: Avoid using plastic lids when working with DIC imaging.

3.2 Place the holder with ‘eggcups’ and observation medium in the microscope. Focus carefully using brightfield light until the ‘eggcups’, and cells are in the same plane of observation.

3.3 Open the software and adjust the parameters. Select the filters TxRed and GFP for actin and myosin; adjust the exposition time for each channel. A typical acquisition rate is 5 s for both channels.

NOTE: The exposition time may have to be adjusted depending on the setup used, dye or other cellular organelles of interest.

3.4 Select the region of interest and seek for a cytokinetic ring using either the GFP or TxRed channel. Focus accurately.

NOTE: The ring is sharper in myosin and easier to recognize.

3.5 Run the automatic acquisition in both channels until the ring is completely closed.

NOTE: Some photobleaching may be observed. Adjust the microscope parameters in order to reduce it.

4. Observation of fixed cellular organelles into the ‘eggcups’

This step can be performed before or after step #3. Cells can be directly fixed after the centrifugation step and stained for the organelle of interest or after the observation in the microscope. This example shows the staining of the Golgi apparatus, nucleus and actin fibers on NIH3T3 fibroblasts in ‘eggcups’.

4.1 Fixation of cells in the ‘eggcups’

4.1.1 Prepare 3 % paraformaldehyde (PFA) and warm at 37 ^{o}C. Remove the ‘eggcups’ sample from the 50 ml tube (or the microscope holder) and place it inside a P35 Petri dish. Rinse once with PBS 1X.

NOTE: Protocols for the preparation of 3 % paraformaldehyde are widely available elsewhere.

CAUTION: Use nitrile gloves and eye protection during the preparation of PFA.

4.1.2 Remove completely the PBS and drop 1 ml of 3 % PFA and incubate for 17 min. Remove the PFA and rinse twice with 1 ml of PBS 1X. Permeabilize cells using 1 ml of 0.5 % Triton for 3 min and wash twice with PBS 1X for 5 min.

4.2 Staining of cells in 'eggcups'

4.2.1 Incubate cells for Golgi apparatus staining with the primary antibody rabbit polyclonal anti-Giantin in a 1:500 dilution in PBS. Place a 100 µl drop of antibody solution onto a plastic film sheet and incubate the cells inside the 'eggcups' upside down for 45 min.

CAUTION: Protect the sample with a cover to prevent drying.

4.2.2 Release carefully the 'eggcups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1X.

4.2.3 Prepare a cocktail in PBS with the secondary antibody Cy3 goat anti-rabbit (1:1000) and with Phalloidin Alexa Fluor 488 (1:200) for staining actin stress fibers.

4.2.4 Incubate cells with a 100 µl drop of antibody solution onto a plastic film sheet and incubate cells inside the 'eggcups' upside down for 45 min.

4.2.5 Release carefully the 'eggcups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1X.

4.2.6 Incubate cells for nucleus staining placing a 100 µl drop of 1 µg ml^{-1} DAPI in PBS onto a plastic film sheet and incubate cells inside the 'eggcups' upside down for 45 min. This step can be performed with step 4.2.3.

4.2.7 Release carefully the 'eggcups' and place them into a P35 Petri dish. Rinse 3 times, 5 min each, with PBS 1X.

4.2.8 Mount cells using a 15 µl Glycerol:PBS (1:1 v/v) on a standard microscope glass slide and seal the sample with nail polish to avoid drying.

NOTE: Depending on the 'eggcups' thickness, mounting may be difficult. It is recommended to store then the sample into a P35 Petri dish in PBS, protected from drying.

4.3 Microscope observation

For this example an upright confocal microscope is used, equipped with PMT and Hybrid detectors. A 25X or 63X HCX IR APO L water objective (0.95 NA,) was selected to provide a wide field of the sample and show the applicability of the device for high-content-screening applications.

4.3.1 Select the 25X or 63X water objective.

NOTE: Different objectives can be used depending on the application and signal. But usage of high numerical aperture objectives is recommended.

4.3.2 Place the fixed sample with the EC and focus carefully using brightfield light (phase contrast or DIC) until the EC and cells are in the plane of observation.

4.3.3 Open the software and adjust the parameters. Select the filters GFP, Cy3 and DAPI for actin, Golgi and nucleus observation, respectively; adjust the exposition time for all channels.

NOTE: The exposition time may have to be adjusted depending on the setup.

4.3.4 Select and focus the region of interest; start image capture (see Figure 3).

5. Adaptation for the observation of yeast cells and *C. elegans* embryo

5.1 Fission and Budding Yeast cells

This example uses fission yeast cells which are tagged with RLC1-mcherry and CHD-GFP for myosin and actin, respectively. The budding yeast cells are not fluorescently labeled here. For fission yeast observation an inverted spinning disk confocal microscope was used. A 100X HCX PL APO CS oil objective (1.4 NA) was used for all acquisitions. Alternatively, cells were also observed using an inverted phase-contrast microscope equipped with a 20X phase contrast air objective LCPlanFl (0.4 NA). In this example, the protocol is identical for both cell types.

5.1.1 Prepare the 'eggcups' surface as described above. For fission and budding yeast cells, prepare cavities of 5 µm in diameter (see Table 1). In this case, the surface does not need to be functionalized with adhesion proteins.

NOTE: The filling can be optimized by using conical 'eggcups'. This shape capture and retain the cells avoiding releasing during the rinsing step after centrifugation. Filling percentage is optimum (about 80 %). These conical 'eggcups' can be fabricated by means of Deep Reactive Ion Etching technology in a specialized microfabrication center [13].

5.1.2 Culture yeast cells in the proper culture media (see Table 1) until reaching an optical density (OD) in the range of 0.2 and 0.8. Sonicate the culture of yeast cells to remove aggregates.

5.1.3 Insert yeast cells in 'eggcups' by centrifugation. For centrifugation, 4 ml of cultured cells in the appropriate OD is added onto the tube with 'eggcups'. After the first centrifugation, gently shake the tube to re-suspend cells which are not in the 'eggcups', while cells in the 'eggcups' are not disturbed. Without opening the tube, centrifuge again and repeat this step twice. This ensures the deposition of cells from the culture into the empty microcavities and will increase the filling percentage.

NOTE: When working with yeast, it is recommended to pre-heat the centrifuge to the working temperature during experiments

NOTE: The protocol can be paused here and continued up to 12 h later. In this case, store the sample at the working temperature and cover it to prevent evaporation.

5.1.4 Place ‘eggcups’ in a microscope holder and fill the holder with filter sterilized medium for imaging. Now rinse the cells with the same media until the floating yeast cells are removed efficiently. Take care not to disturb cells in ‘eggcups’ during the rinsing process.

5.1.5. Select the 100X oil objective and focus carefully. Open the software and adjust the parameters. For fission yeast, select the filters GFP and TxRed for actin and myosin and adjust the exposition time for both channels. A typical acquisition rate is 3 s. Start image acquisition.

NOTE: Depending on the fluorophore, type of tagging and the set-up, exposure time varies for other systems.

5.2 *C. elegans* Embryo

This example uses *C. elegans* embryos 25-30 µm wide and 50-55 µm long. Embryos were cultured as indicated in [24]. A simple visual protocol of how to manipulate *C. elegans* can be found in [25]. The observation was performed using an inverted phase-contrast microscope equipped with a 40X air objective 0.55 NA.

5.2.1 Prepare the ‘eggcups’ surface as described above and 25 µm in diameter (see Table 1). In this case, the surface does not need to be functionalized with adhesion proteins.

5.2.2 Culture the *C. elegans* embryos in the proper culture media (see Table 1).

5.2.3 Insert embryos in ‘eggcups’ by centrifugation as described above (see section 2.6 to 2.12) using ultrapure water as culture medium.

NOTE: Embryos were ‘behaving’ normally in ultrapure water for the duration of the experiment. Alternatively use a physiological M9 buffer for long-term experiments.

5.2.4 Rinse the sample as described above (see section 2.14). Place the ‘eggcups’ into a microscope holder. Select the 40X air objective and focus carefully. Open the software and adjust the parameters. Select an acquisition rate of 3 s. Start image acquisition.

Representative Results:

The ‘eggcups’ are a novel high-content-screening methodology which allows the visualization of oriented cells and embryos in a 3D environment. Additionally, some cellular processes, which are difficult to observe in standard 2D (flat) cultures, can be observed by this new method. Figure 1(a) shows a summary of the procedure for the EC microfabrication (see also Section 1 in the above-described protocol). The method is simple, fast, efficient and without any requirement of special equipment. Figure 1(b) and (c) show a large-scale picture and a magnified scanning electron microscope image of ‘eggcups’, respectively. As it can be observed, their shape and size are very regular. This method is very flexible; different shapes and sizes can easily be fabricated and adapted for different model systems. The dimensions of ´eggcups´ were selected in the following manner: dimensions of cells which undergo division were measured on 2D surfaces: they have a spherical shape and their diameter was taken as a good indication for the EC diameter. Cells in ´eggcups´ elongate and orient along their long axis

during cell division for example. This dimension depends on the system - cells and embryos - so this dimension should be evaluated in each case.

Figure 2 shows the material needed (Figure 2a) and a step-by-step protocol (Figure 2b) about how to use the 'eggcups' (see also Section 2 in the above-described protocol). The filling of the EC with cells of interest (or other model systems) is very simple and fast. Typically, it takes less than 20-30 min, which also includes the time for cell trypsinization. After the filling, samples can be used to study active processes (live imaging) or can be fixed and stained for the visualization of organelles of interest (see also Sections 3 and 4 in the protocol described above).

On flat surfaces, cells show heterogeneous responses and extreme phenotypes of cellular organelles. In fact, it has been suggested that actin stress fibers (and other cellular organelles) are artifacts of the culture conditions [1]. In order to prove this hypothesis, we cultured NIH3T3 cells both on 3D 'eggcups' and on flat surfaces and compared the phenotypes of different cellular organelles, namely actin stress fibers, Golgi apparatus and nuclei. Figure 3 shows an example of how cells are organized on both configurations. In EC, cells are distributed in an ordered array showing a homogeneous spherical-like phenotype (Figure 3a). On flat surfaces, cells show the typical disordered, spread and heterogeneous morphology (Figure 3b). There are also significant differences in cytoskeleton structures. In particular, cells on 'eggcups' show a reduction in the number of stress fibers compared to flat surfaces. This is further confirmed in the 3D reconstructed images where no clear stress fibers are visible (see Figure 3c-d). This confirms that some cellular structures are magnified in 2D cultures. This is also in agreement with observations performed *in vivo* where stress fibers cannot be identified.

The Golgi apparatus also shows significant variation in their phenotype depending on the culture condition (see Figure 4). The Golgi apparatus on 2D cultures typically shows an extended phenotype 'embracing' the nucleus periphery whereas in 'eggcups' it shows a more compacted phenotype (see Figure 4a-b). In order to simulate a drug screening manipulation, we also evaluated the effect of drugs on cells cultured on both environments. We selected Blebbistatin mainly because it disrupts the actin stress fibers and could have an effect on Golgi morphology (see Figure 3c-d). Since the Golgi is located next to the cell nucleus, this drug could also have an effect on its architecture. We first observed that cells treated with this drug showed a less regular and uniform morphology compared to wild-type (WT) cells (see Figure 3c-d). We then compared and quantified the Golgi phenotype observed on 'eggcups' and on flat surfaces (see Figure 4c). We observed that on 2D surfaces cells showed mostly an extended phenotype whereas on 'eggcups' cells showed a more compacted phenotype. We did not observe though a striking difference between WT and Blebbistatin-treated cells.

Finally, on 2D surfaces the cell nucleus is randomly oriented whereas cells on EC it is orthogonally oriented on the XY plane on both WT and Blebbistatin-treated cells (see Figure 5 a-c). This highlights the strength of the device to orient cellular organelles, similar to a former application of the method for orienting the plane of observation of the cytokinetic ring in yeast and mammalian cells [10,12,13]. We finally studied how the nucleus sphericity (defined as $\Psi=[\pi^{1/3} 6V_n^{2/3}]/A_n$, where V_n is the volume of the nucleus and A_n its surface area) was affected depending on the culturing condition and upon the treatment of cells with Blebbistatin. Figure

5d shows the corresponding distributions of Ψ. We did not observe a difference for WT^{flat} *vs* WT^{EC}, which reveals that the EC are not affecting the normal sphericity of cells. However, we observed a difference when comparing WT^{EC} to $Blebb^{EC}$ suggesting that the EC are revealing a real effect of the drug that is masked in 2D.

Live cell studies using 'eggcups' allow also identification of novel active processes which are not visible in standard cultures. We plated cells in EC and visualized cell division. Figure 6 shows a sequence of images of the cytokinetic ring closure during cell mitosis. The 'eggcups' device allows a complete visualization of the ring, whereas standard 2D cultures only shows 2 spots which corresponds to one single plane [10]. Reconstruction of the ring from a sequence of z-stack images using 2D cultures can be done [26], but important information is lost. The quality is diminished due to low z resolution and dynamic processes cannot be resolved. Actin and myosin are the key proteins in the force generation of cell division. Their dynamics cannot be imaged and studied in 2D culture (Figure 6a), whereas with 'eggcups' it is immediately revealed. We have identified novel structures and processes: in HeLa cells we find periodic accumulations of myosin. These accumulations move radially as the ring is closing (Figure 6b). In fission yeast we also find in-homogeneities in myosin and actin (Figure 6c, *right*). In contrast to what we see in HeLa cells, they rotate on the ring during closure. The speed is in the range of µm min^{-1} and would not be resolvable by z reconstruction with standard microscopes. Finally the cytokinetic ring can be further studied by staining for its components. We find that there is an accumulation of phosphotyrosine in the vicinity of the ring (Figure 6d). We can also show that anillin is colocalizing in the ring with myosin and actin (Figure 6e). By staining the cells in this orientation, we reveal that anillin shows also an inhomogeneous distribution, which is similar to the distribution of myosin.

The 'eggcups' were also applied to different model system: we reported mammalian cells, fission yeast, but we also tested budding yeast and *C. elegans* (see Figure 7a-e). In this case, the protocol was adapted for each specific system in terms of culture media, cavities size and morphology (see Table 1). As an example, conical *V*-shaped 'eggcups' were the optimal morphology for immobilizing fission yeast efficiently [12], instead of completely cylindrical (or U-shaped) shape used for mammalian cells [13]. This allowed testing the effect of different cytoskeleton drugs with potential application in Life Science research. This demonstrates the flexibility and reliability of the developed methodology.

Furthermore, the highly ordered arrangement of cells allows an easy, automated read-out of the fluorescence of single cells. We illustrate this by inserting NIH3T3 cells expressing GFP in 'eggcups' (Figure 8a). The cell position can easily been recognized and the corresponding expression level measured. Figure 8b shows the distribution of fluorescence signals. This can be applied to any read-out (immunofluorescence, fluorescent reporters in cells for example).

Tables and Figures:

Figure 1. Fabrication of 'eggcups'. (a) Schematic description of the fabrication procedure of 'eggcups' by replica molding: (i) Pour liquid PDMS on the SU-8 mold and cure it. (ii) Cut out the stamp and remove it carefully from the surface, then plasma activate it to silanize it. (iii) Pour liquid PDMS on the silanized stamp and centrifuge it to obtain a thin PDMS layer. (iv) After curing the PDMS layer, plasma activate both, the PDMS covered stamp and a glass coverslip. (v) Plasma bind both by applying a gentle, homogeneous pressure. (vi) After plasma bonding, remove carefully the stamp to uncover the 'eggcups' surface. (vii) To simplify the handling in the next steps, add a small PDMS handle piece. Bind the PDMS piece to the coverslip by gluing it with liquid PDMS and (viii) cure it then in the oven. (b) Image of a 25 mm coverslip with PDMS 'eggcups' and a handle. c) Scanning electron microscope images of PDMS 'eggcups'. The distance between centers of 'eggcups' is 30 µm, and their diameter about 25 µm. *(Left)* Top view. *(Right)* 'eggcups' are cut to image the inner part.

Figure 2. (a) Elements needed for the EC filling. (1) 50 ml tube; (2) cylindrical piece (top and side view); (3) cell culture medium; (4) 'eggcups'; (5) sharp tweezers. (b) Schematic of the EC filling procedure. A cylindrical piece is first introduced into a 50 ml tube and filled with 13 ml of cell culture medium (i). Next, (ii) the 'eggcups' are gently deposited on top of the cylindrical piece using sharp tweezers to manipulate the EC using the small PDMS piece. (iii) Cells at the proper density are pipetted on top of the EC. (iv) Cells are introduced in the 'eggcups' by centrifugation. (v) Finally, the sample is gently released out from the tube and it is ready to use.

Figure 3. Comparison of cell phenotypes on 3D 'eggcups' and 2D flat surfaces. Confocal microscopy (25X water objective, 0.95 NA, Leica) image of NIH3T3 cells on (a) EC forming an ordered array, and showing a homogeneous spherical phenotype, and on (b) standard 2D flat culture, randomly distributed with heterogeneous phenotypes. Cells were stained for actin (in green), Golgi (in orange) and nucleus (in blue). Scale bars: 100 µm. (c) 3D reconstruction of cells on EC and (d) on flat surfaces for WT and Blebbistatin-treated cells. Scale bars: 20 µm.

Figure 4. Study of NIH3T3 Golgi apparatus phenotype. Schematic and sample image of Golgi phenotype classification for cells on (a) flat and (b) EC. Cells were classified as compacted, extended or fragmented depending on the α-value. (c) Quantification of Golgi phenotypes. Scale bars: 10 µm.

Figure 5. Study of NIH3T3 nucleus phenotype. (a) *(Left)* Confocal microscopy image of a NIH3T3 cell inside an EC and stained for actin (in green), Golgi (in orange) and nucleus (in blue). *(Right)* Scheme of nuclei orientation inside EC. (b) Angular distribution of nuclei inside EC for WT and (c) Blebbistatin-treated cells. (d) Nucleus sphericity values for WT and Blebbistatin-treated cells both for EC and flat surfaces ($P[WT^{EC}\text{-}Blebb^{EC}]<0.001$, $P[Blebb^{flat}\text{-}Blebb^{EC}]<000.1$; $n_{WT}{}^{flat}=47$, $n_{WT}{}^{EC}=94$, $n_{Blebb}{}^{flat}=59$, $n_{Blebb}{}^{EC}=141$ cells). Scale bar: 10 µm.

Figure 6. Detailed study of the cytokinetic ring in live and fixed samples and in two systems using 'eggcups'. (a) Time sequence of the cytokinetic ring using standard 2D *in vitro* culture.

Only two bright spots in actin (Lifeact-mcherry, red) and myosin (GFP tagged, green) are visible in the cleavage furrow of the HeLa cells (Scale bar: 10 µm). (b) Time sequence of the closure for the cytokinetic ring in HeLa cells during mitosis using 'eggcups'. The images show actin (in red) and myosin (green). 'Eggcups' allow the identification of still myosin accumulations. One example is highlighted with an arrowhead. (Scale bar: 5 µm). (c) The cytokinetic ring can also be visualized in fission yeast. *(Left*) Cells lie on a flat surface, the cytokinetic ring is only visible as two dots. *(Right)* Cells in 'eggcups': the entire closure can be captured. Actin is labeled with CHD-GFP (Scale bars: 2 µm). Time in min:sec. (d-e) Examples of stained cytokinetic rings. (d) Actin-GFP expressing HeLa cells are stained for phosphotyrosine (PY) which also shows signal in the ring (Scale bar: 5 µm). (e) HeLa cells expressing GFP tagged myosin and Lifeact-mcherry (actin) are stained for anillin. Anillin is revealed to localize in the cytokinetic ring and less concentrated in the cortex. It shows co-localization with actin and myosin (Scale bar: 5 µm).

Figure 7. Application of the 'eggcups' to other cell types and model systems. (a) U2OS (human osteosarcoma). The inset shows a dividing cell. (Scale bar = 20 µm). (b) NIH3T3 cells expressing GFP. Difference in expression levels can be easily read out (Scale bar = 20 µm) (c) SW480 cells (Scale bar = 20 µm). (d) Budding yeast; their cycle time is unchanged. (Scale bar = 10 µm). (e) *C. elegans* worms; *(Left)* on a flat surface. *(Right)* In 'eggcups', embryo is seen from an otherwise hidden perspective. (Scale bars: 10 µm). Time in min:sec.

Figure 8. The organization in an array of 'eggcups' allows an automated analysis of cell population. (a) NIH3T3 cells in EC (Scale bar = 20 µm). They have different expression levels of GFP. (b) Automated recognition of cell position allows an individual analysis of the expression level. It is summarized in the histogram of the GFP expression of the cell population.

Table 1. Culture conditions in 'eggcups' for different model systems. The above-related protocol can easily be adapted by just replacing the described culture conditions and the size of 'eggcups'

Discussion:

Replica molding was used in order to fabricate the 'eggcups'. The fabrication process does not need a clean room; it is easy and simple, although some practice may be required. In particular, releasing the PDMS stamp is the most critical step in order to produce a large area of high quality 'eggcups'. For this reason, special care has to be taken in this step. If this step is repeatedly failing, consider to optimize the plasma cleaner parameters prior to the silanization and plasma binding. Insufficient silanization will lead to strong sticking of the stamp to the PDMS film. If this is observed, the incubation time with the siliconizing reagent can be increased. Note that other techniques and materials can be applied to fabricate the 'eggcups', which can be functionalized with a large range of ligands (fibronectin, gelatin, collagen, etc.). In particular, microcavities in polystyrene can be easily fabricated by custom-made hot-embossing technique. This ensures biocompatibility and direct comparison with results obtained in standard culture dishes. Similarly, special care and practice are required in order to optimize

the filling percentage. In particular, the rinsing step is critical in order to ensure an appropriate filling with no excess of cells, contributing to noise and background in the signal. If cells are removed easily from cavities, consider to change the size or depth of cavities.

'Eggcups' provide 3D-like architecture to cells and high content screening assays using a simple protocol. Cellular organelles and active processes unknown using standard culture assays can be easily visualized by means of inserting single cells on individual microcavities ('eggcups'). Depending on the model system, the size, shape and their dimensions can be easily adapted. In this way mammalian cells, fission yeast, budding yeast and *C. elegans* can be manipulated and studied, as well as any embryos such as Drosophila, mice or human embryos for *in vitro* fertilization, or stem cells for example.

In this setup single cells are captured. This is in contrast to epithelial tissues encountered *in vivo*. However, this environment could be reproduced in our 'eggcups' by coating the side walls with cadherins to mimic cell-cell contacts using more flexible elastomers. Focal contacts will be promoted by the deposition of fibronectin at the bottom of wells. These respective distributions of adhesion molecules should allow in reproducing the cellular environments encountered *in vivo*. By this method one would approach the physiological conditions.

Medium exchange in our assay is ensured. Cells in EC do not show any degradation when performing both short and long-term experiments due to lack of medium exchange. Note also that cells in EC can be cultured until confluence although the main interest is when individual cells or embryos are isolated within the cavities.

Orientation of organelles or entire organisms is revealing new information. We show different dynamics of actin and myosin in the cytokinetic ring. Although the cytokinetic ring in fission yeast and mammalian cells is composed of similar key components, we show with this setup, that their specific dynamics is different. This is supporting the result, that the closure mechanism in the two systems is different as well. To develop and investigate such a hypothesis, the orientation of the cell is indispensible. In future studies, this device can be also used to investigate other events related to organelle organization in cells.

Beyond that, this technique can be of great use in developmental biology. Elongated embryos can be easily oriented, observed or further treated in a defined orientation. Probably our assay would not impose polarity of embryos, but the high filling percentage would allow to extract the desired read-out in a reliable manner. Altogether 'eggcups' could be a good device for high content screenings.

Other culture assays have been proposed. These methods range from multiple cells in 2D dimensions in multiwell plates, to single cells deposited in micropatterned adhesive motifs with identical shape. However, none of them is appropriate to overcome the limitations detailed above on the observation of cellular organelles and dynamical processes [1].

Future improvements to our system will allow the applicability of 'eggcups' to industrial-oriented purposes. As an example, drug screening applications in pharmaceutical companies require the use of multiwell plates [14,27]; implementing 'eggcups' into such platforms will

potentially improve the reliability of tests and results. As such, high-content-screening assays will be performed using the commonly used automatised processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability. Some commercial products based on 3D-cell cultured assays have already appeared in the market highlighting the importance of this kind of assays. Finally, these devices open new perspectives for personalized medicine: cells from patient could be placed in 'eggcups', and treatment cocktails could be tested in a physiological environment; the biomarker read-out will allow to anticipate an optimal treatment to be given to the patient [28]. Altogether the physical shape of the cells and embryos are guiding the architecture of the cavities, and we hope that the device and this method will be widely spread in the future.

Acknowledgments: We acknowledge L. Brino (IGBMC High Content Screening facility, Illkirch, France) for providing us with the anti-Giantin antibody, M. Labouesse Lab. for *C. elegans* (IGBMC) and B. Séraphin Lab. for budding yeast (IGBMC), E. Paluch and A. Hyman for fluorescent HeLa cells (MPI-CBG, Dresden), J. Moseley (Dartmouth Medical School) and J.Q. Wu (Ohio State University) for fission yeast cells; A. Hoël and F. Evenou for experimental help, C. Rick (IBMC, Strasbourg, France) for technical help, and J.C. Jeannot (Femto-st, France) for help in microfabrication. This work was supported by funds from the CNRS, the University of Strasbourg, Conectus, La Fondation pour la Recherche Médicale and the ci-FRC of Strasbourg.

Disclosures: We have nothing to disclose.

References:

1 Cukierman, E., Pankov, R., Stevens, D. R. & Yamada, K. M. Taking Cell-Matrix Adhesions to the Third Dimension. *Science* **294**, 1708-1712 (2001).

2 Azioune, A., Storch, M., Bornens, M., Thery, M. & Piel, M. Simple and rapid process for single cell micro-patterning. *Lab Chip* **9**, 1640-1642 (2009).

3 Mandal, K., Balland, M. & Bureau, L. Thermoresponsive Micropatterned Substrates for Single Cell Studies. *PLoS ONE* **7**, e37548 (2012).

4 Pampaloni, F., Reynaud, E. G. & Stelzer, E. H. K. The third dimension bridges the gap between cell culture and live tissue. *Nat Rev Mol Cell Biol* **8**, 839-845 (2007).

5 Baker, B. M. & Chen, C. S. Deconstructing the third dimension: how 3D culture microenvironments alter cellular cues. *J Cell Sci* (2012).

6 Ghibaudo, M., Di Meglio, J.-M., Hersen, P. & Ladoux, B. Mechanics of cell spreading within 3D-micropatterned environments. *Lab Chip* **11**, 805-812 (2010).

7 Greiner, A. M., Richter, B. & Bastmeyer, M. Micro-Engineered 3D Scaffolds for Cell Culture Studies. *Macromol Biosci* **12**, 1301-1314 (2012).

8 Khetan, S. *et al.* Degradation-mediated cellular traction directs stem cell fate in covalently crosslinked three-dimensional hydrogels. *Nat Mater* **12**, 458-465 (2013).

9 Legant, W. R. *et al.* Measurement of mechanical tractions exerted by cells in three-dimensional matrices. *Nat Meth* **7**, 969-971 (2010).
10 Riveline, D. & Buguin, A. Devices and methods for observing the cell division. WO/2010/092116 (2010).
11 Ochsner, M. *et al.* Micro-well arrays for 3D shape control and high resolution analysis of single cells. *Lab Chip* **7**, 1074-1077 (2007).
12 Riveline, D. Devices and methods for observing cells with cell wall or invertebrate embryos with oblong eggshell WO 2013144302 A1 (2012).
13 Riveline, D. & Wollrab, V. Devices and methods for observing eukaryotic cells without cell wall. WO 2013135809 A1 (2012).
14 Zanella, F., Lorens, J. B. & Link, W. High content screening: seeing is believing. *Trends Biotech* **28**, 237-245 (2010).
15 Wolfe, D., Qin, D. & Whitesides, G. in *Microengineering in Biotechnology* Vol. 583 *Methods in Molecular Biology* eds Michael P. Hughes & Kai F. Hoettges) Ch. 3, 81-107 (Humana Press, 2010).
16 Mehling, M. & Tay, S. Microfluidic cell culture. *Curr Op Biotech* **25**, 95-102 (2014).
17 Yao, X. *et al.* Functional analysis of single cells identifies a rare subset of circulating tumor cells with malignant traits. *Integr Biol* **6**, 388-398 (2014).
18 Eberwine, J., Sul, J.-Y., Bartfai, T. & Kim, J. The promise of single-cell sequencing. *Nat Meth* **11**, 25-27 (2014).
19 Allen, T. D. *et al.* Generation of cell-free extracts of Xenopus eggs and demembranated sperm chromatin for the assembly and isolation of in vitro-formed nuclei for Western blotting and scanning electron microscopy (SEM). *Nat. Protocols* **2**, 1173-1179 (2007).
20 Robbins, E. & Marcus, P. I. Mitotically Synchronized Mammalian Cells: a Simple Method for Obtaining Large Populations. *Science* **144** (1964).
21 Whitfield, M. L. *et al.* Stem-loop binding protein, the protein that binds the 3' end of histone mRNA, is cell cycle regulated by both translational and posttranslational mechanisms. *Molecular and Cellular Biology* **20** (2000).
22 Straight, A. F. *et al.* Dissecting Temporal and Spatial Control of Cytokinesis with a Myosin II Inhibitor. *Science* **299**, 1743-1747 (2003).
23 Tang, J., Erikson, R. L. & Liu, X. Checkpoint kinase 1 (Chk1) is required for mitotic progression through negative regulation of polo-like kinase 1 (Plk1). *Proc Natl Acad Sci* **103**, 11964-11969 (2006).
24 Zahreddine, H., Zhang, H., Diogon, M., Nagamatsu, Y. & Labouesse, M. CRT-1/Calreticulin and the E3 Ligase EEL-1/HUWE1 Control Hemidesmosome Maturation in C. elegans Development. *Curr Biol* **20**, 322-327 (2010).
25 Porta-de-la-Riva, M., Fontrodona, L., Villanueva, A., Cer & n, J. Basic Caenorhabditis elegans Methods: Synchronization and Observation. *JoVE*, e4019 (2012).
26 Saha, S. & Pollard, T. D. Anillin-related protein Mid1p coordinates the assembly of the cytokinetic contractile ring in fission yeast. *Mol Biol Cell* **23**, 3982-3992 (2012).
27 Prestwich, G. D. Evaluating Drug Efficacy and Toxicology in Three Dimensions: Using Synthetic Extracellular Matrices in Drug Discovery. *Acc Chem Res* **41**, 139-148 (2007).
28 Futamura, Y. *et al.* Morphobase, an Encyclopedic Cell Morphology Database, and Its Use for Drug Target Identification. *Chem Biol* **19**, 1620-1630 (2012).

Figure1
Click here to download Figure: Wollrab_et_al_Figure1.eps

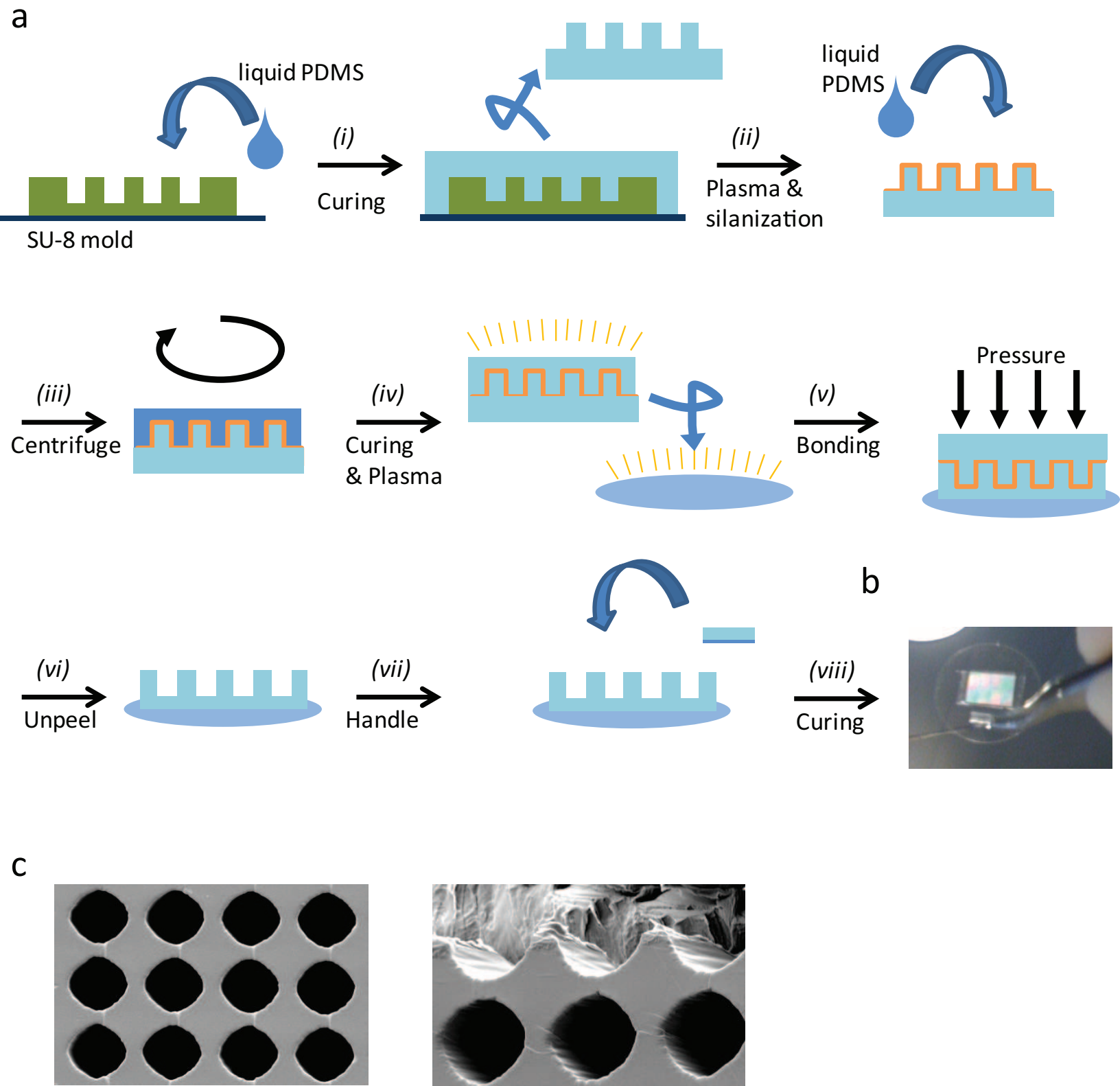


Figure2
Click here to download Figure: Wollrab_et_al_Figure2.eps

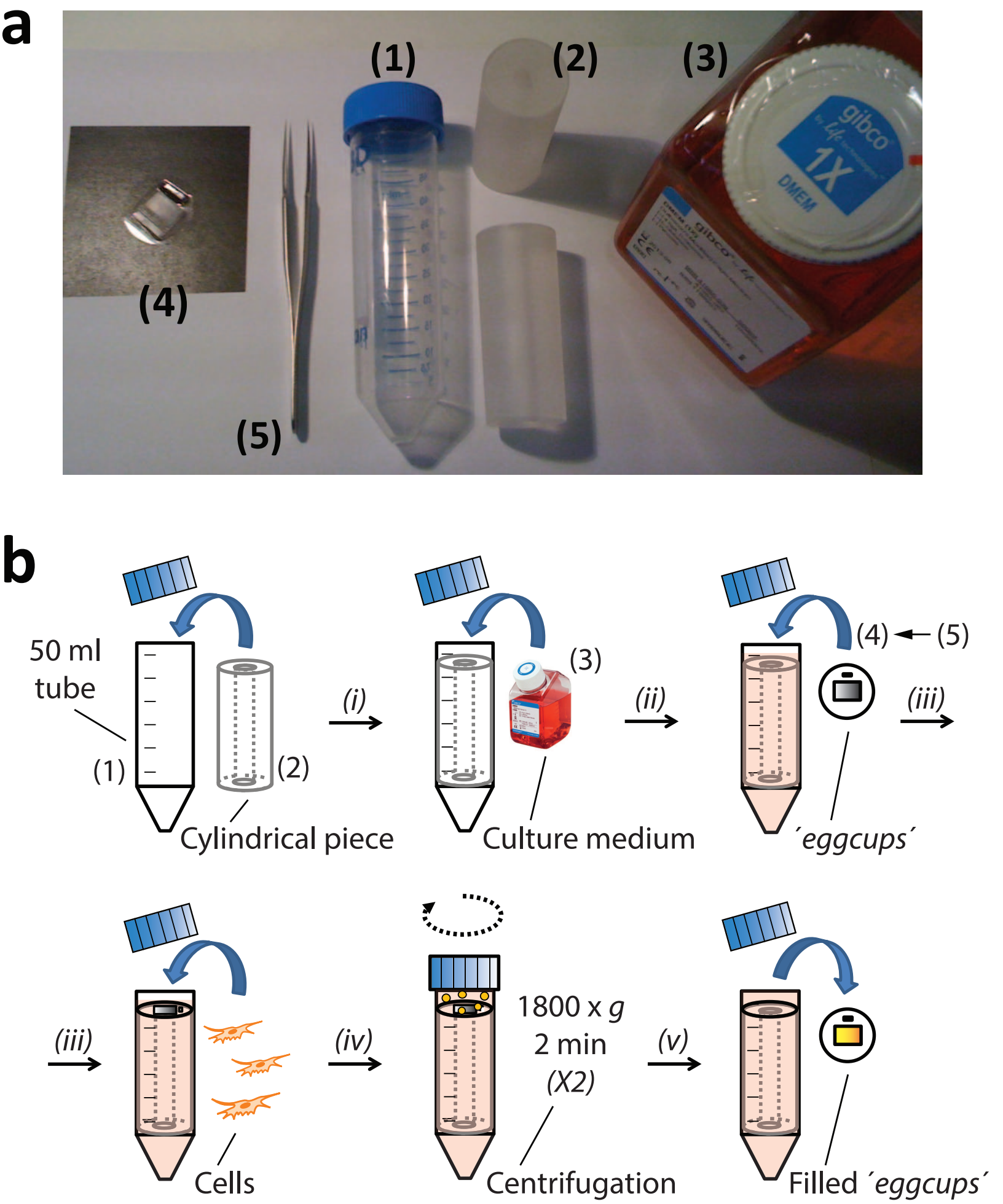


Figure3
Click here to download Figure: Wollrab_et_al_Figure3.eps

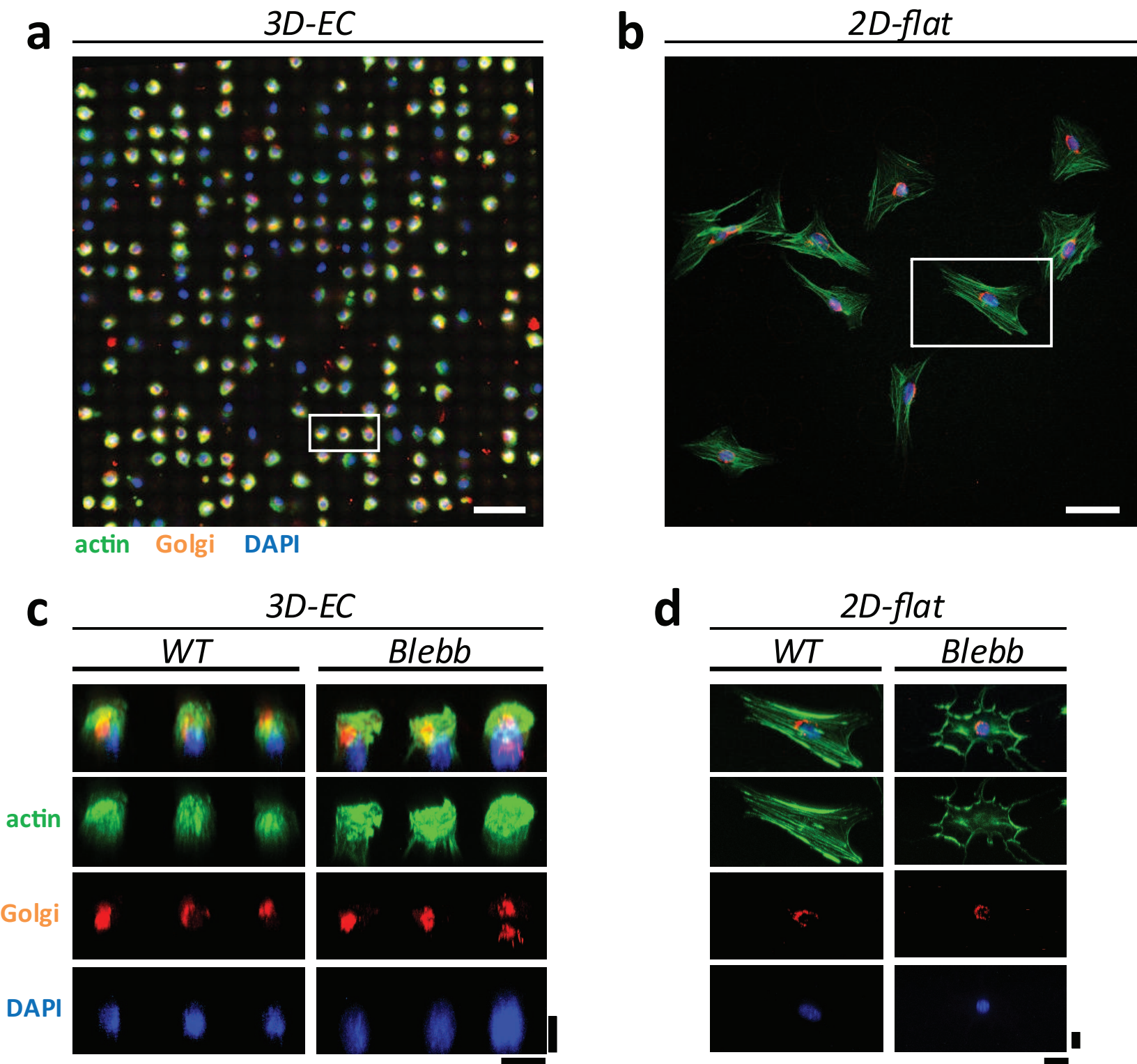
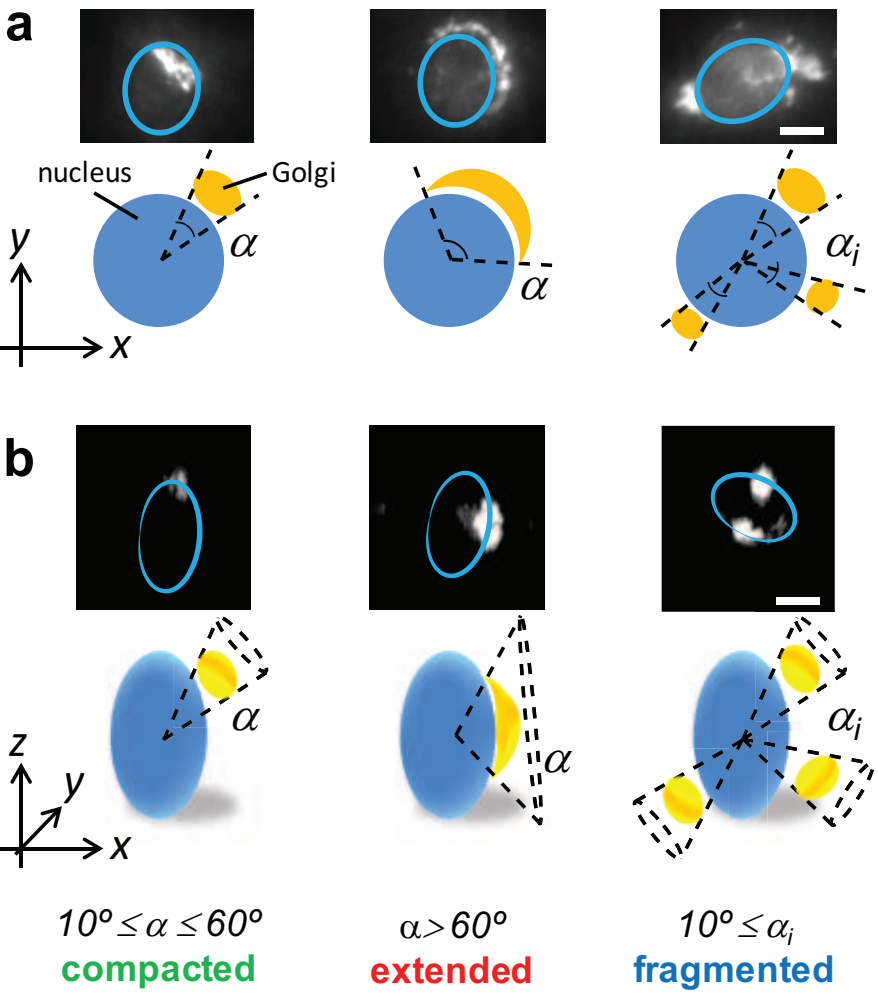


Figure4
Click here to download Figure: Wollrab_et_al_Figure4.eps



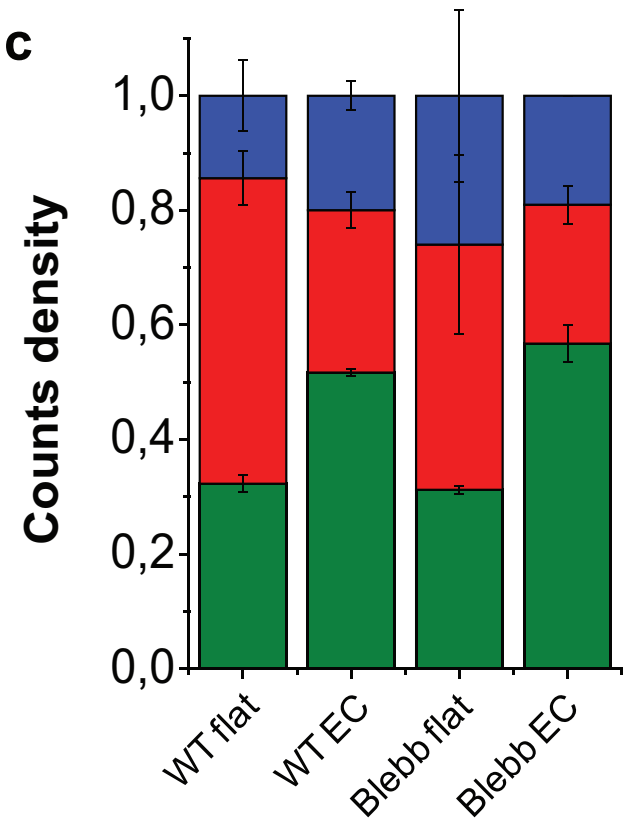


Figure5
Click here to download Figure: Wollrab_et_al_Figure5.eps

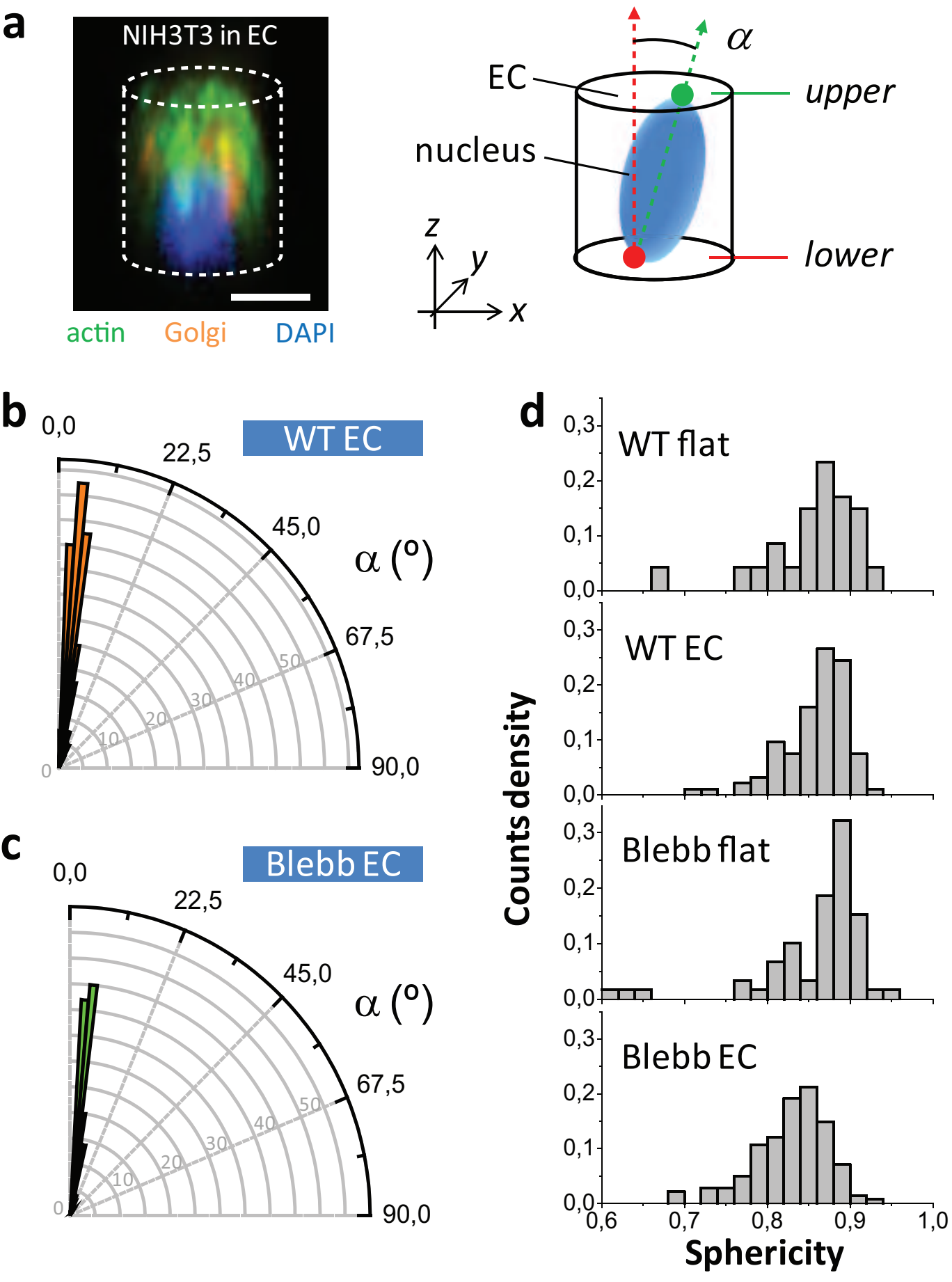


Figure6
Click here to download Figure: Wollrab_et_al_Figure6.eps

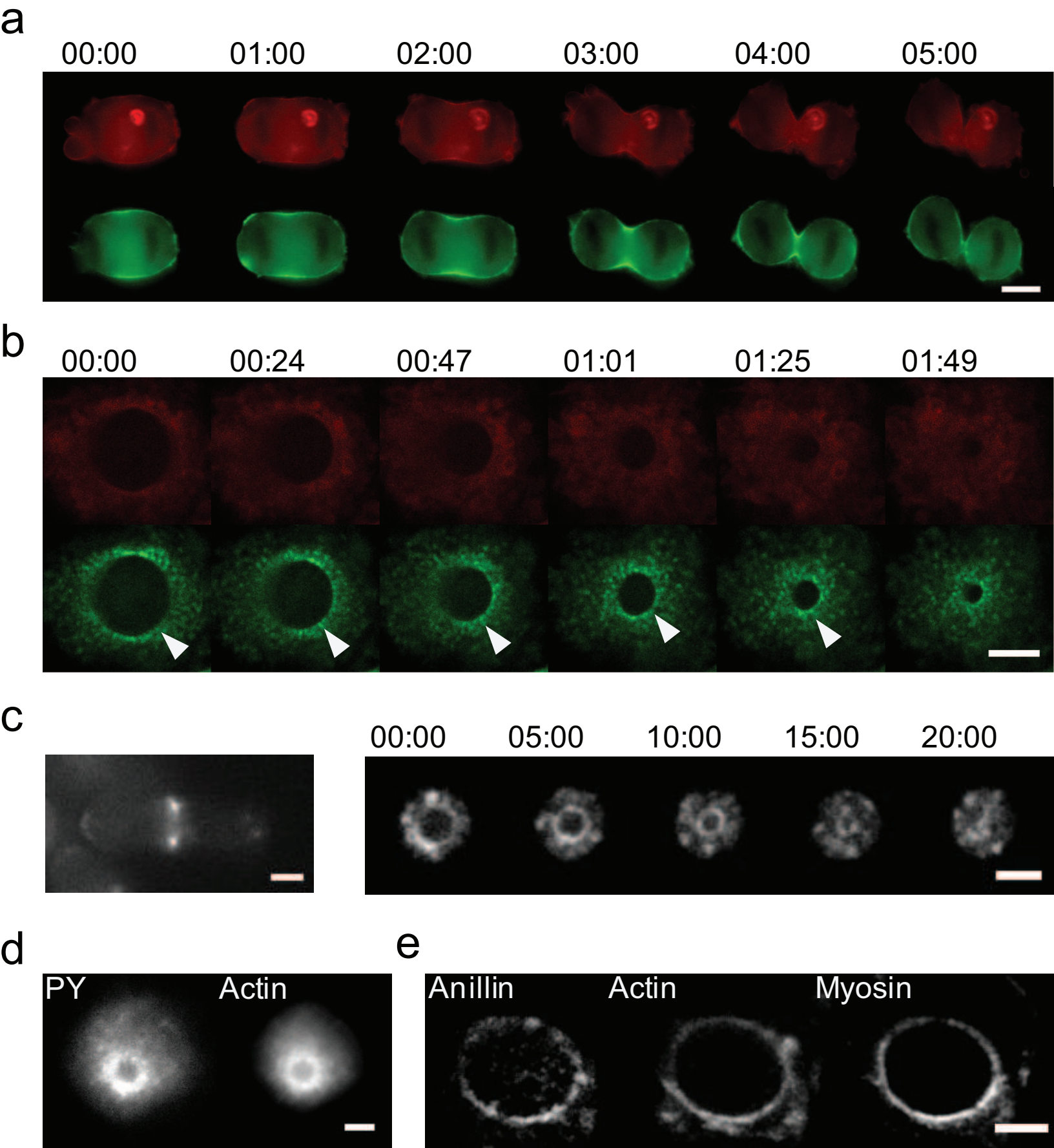


Figure7
Click here to download Figure: Wollrab_et_al_Figure7.eps

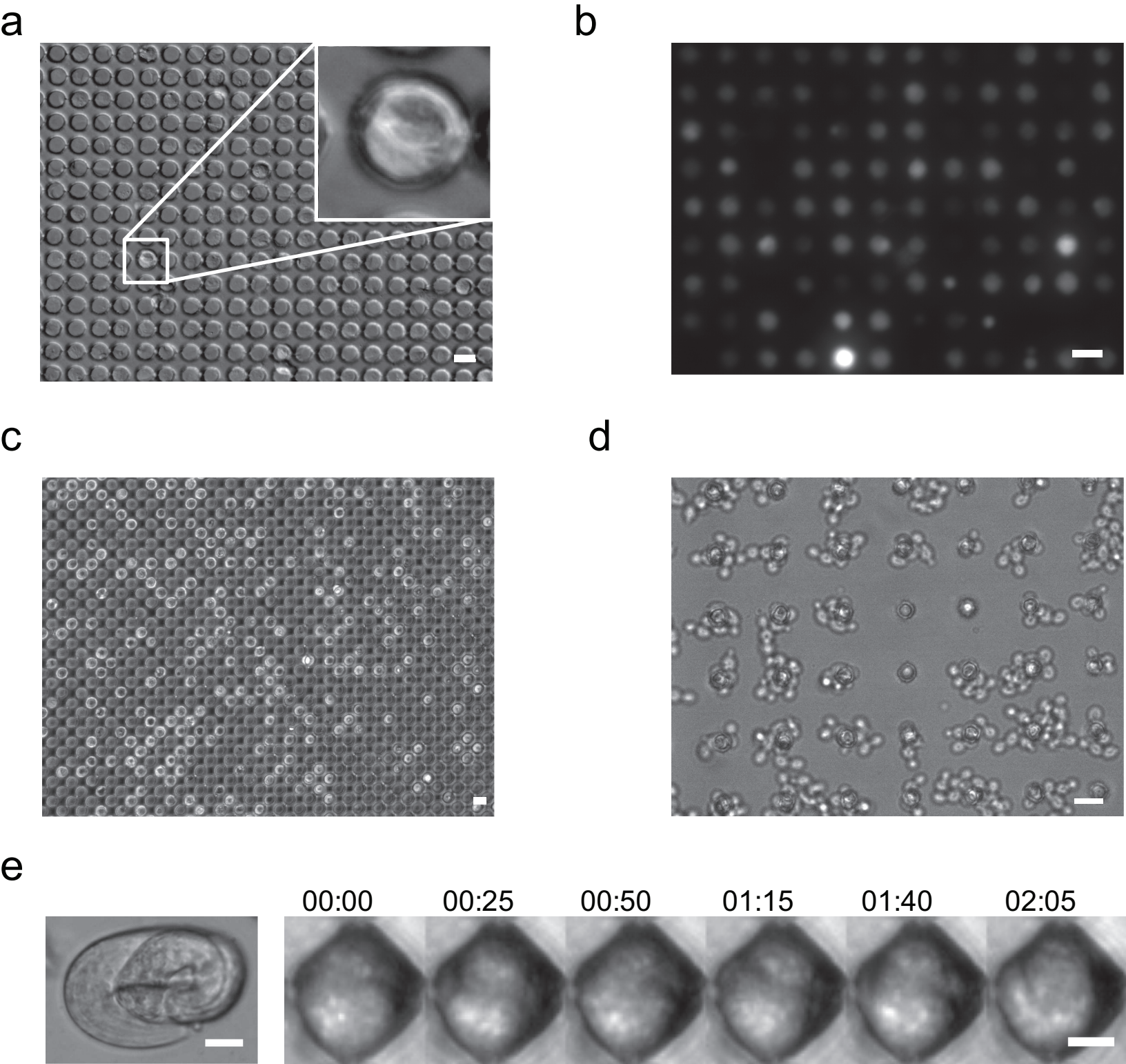
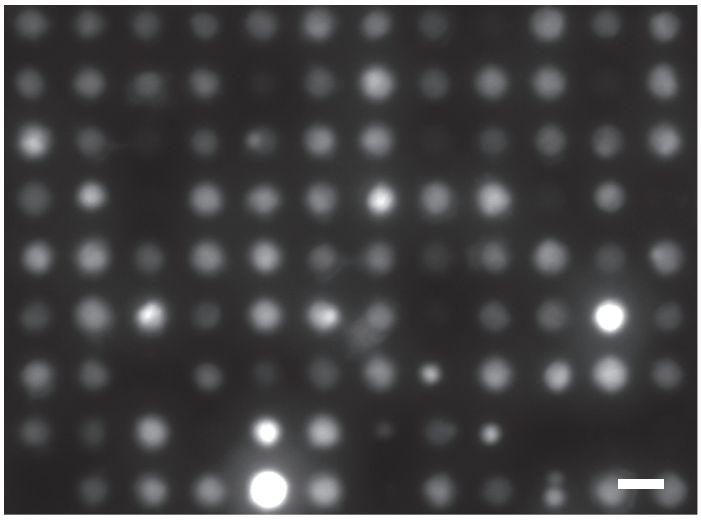


Figure8
Click here to download Figure: Wollrab_et_al_Figure8.eps

a



b

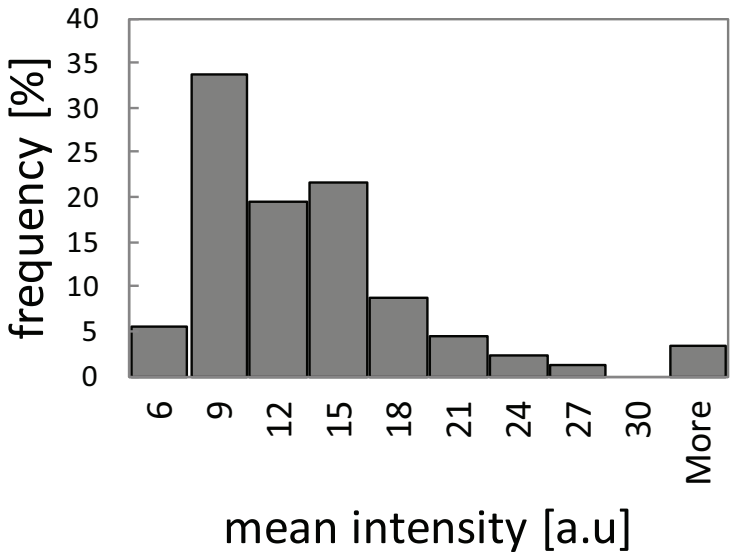


Table1

Click here to download Table: Wollrab_et_al_Table1.xlsx

Model System	Type
Mammalian cells	NIH3T3
	HeLa
	U2OS
	SW480
Yeast	Fission Yeast
	Budding Yeast
Embryo	*C. elegans*

Culture Medium

10 % BCS high-glucose DMEM
10 % FCS high-glucose DMEM
10 % FCS high-glucose DMEM
10 % FCS high-glucose DMEM

Agar plate (YE5S) and liquid media (YE5S and EMM5S)
Agar plate (YPD) and liquid media (YEPD and SD)

NGM plate

Observation Medium	'eggcups' diameter (µm)
10 % BCS L-15	20
10 % FCS L-15	25
10 % FCS L-15	20-25
10 % FCS L-15	17-20
Filter sterilized EMM media (see the list of materials)	5
SD media	5
ultrapure water	25

Comments/Description

Other stable cell lines, such as REF52 or MDCK, as well as primary cell lines, cancerous cells and/or stem cells can alsc
Available from many different sources.
Available from many different sources.
Available from many different sources.

The surface does not need to be functionalized with adhesive proteins.
The surface does not need to be functionalized with adhesive proteins.

Alternatively M9 medium can be used for long-term experiments. The recipe of this salted solution can be found here

ɔ be inserted in the 'eggcups'.

ɛ: http://cshprotocols.cshlp.org/content/2009/5/pdb.rec11798.full?text_only=true

Table of Materials

Click here to download Table: Wollrab_et_al_Materials.xlsx

Name of Material	Company	Catalog Number	Comments/Description
ddH_20 (ultrapure)	Millipore	-	Use always fresh water.
Parafilm (plastic film)	Bemis	PM-999	Adhere Parafilm to the lab bench using some water droplet
Photo-mask	Selba	-	http://www.selba.ch
Silicon wafer	Siltronix	-	http://www.siltronix.com/
			http://www.microchem.com/Prod-SU82000.htm
SU-8 photoresist	MicroChem	2000 series	working in a fumehood is required; check the data sheet f
			http://microchem.com/Prod-Ancillaries.htm
SU-8 developer	MicroChem	-	working in a fumehood is required; check the data sheet f
			http://www.sigmaaldrich.com/catalog/product/sial/i9030
2-propanol	Sigma-Aldrich	19030	Available from multiple companies.
			http://www.sigmaaldrich.com/catalog/product/sigma/sl2
Sigmacote (siliconizing reagent)	Sigma-Aldrich	SL2-25ML	harmful, working in a fumehood is required; check the dat
Chlorotrimethylsilane (TMCS)	Sigma-Aldrich	386529-100ML	http://www.sigmaaldrich.com/catalog/product/aldrich/38
			TMCS produces acute inhalation and dermal toxicity, and i
			http://www.kcprofessional.com/products/ppe/hand-glove
Nitrile gloves	Kleenguard	57372	Available from multiple companies.
			http://www.knittelglass.com/index_e.htm
Glass coverslips #0	Knittel glass	KN00010022593	Very fragile. Manipulate gently.
Sharp straight tweezers	SPI	0WSSS-XD	http://www.2spi.com/catalog/tweezers/t/elec7
			http://www.bdbiosciences.com/cellculture/tubes/feature
50 ml tube	BD Falcon	352070	Available from multiple companies.
			http://www.dowcorning.com/applications/search/default
PDMS	Dow Corning	Sylgard 184 kit	The package contains both PDMS base and curing agent. S
			http://www.dutscher.com/frontoffice/search
Microscope glass slides	Dutscher	100001	Available from multiple companies.
DMEM high-glucose medium	Fisher Scientific	41965-039	http://www.fishersci.com/ecomm/servlet/Search?LBCID=
Bovine calf serum	Sigma-Aldrich	C8056-500ML	http://www.sigmaaldrich.com/catalog/product/sigma/c80
0,25% Trypsin-EDTA	Fisher Scientific	25200-072	http://www.fishersci.com/ecomm/servlet/Search?keyWo
			http://www.fishersci.com/ecomm/servlet/Search?keyWo
PBS 1X	Fisher Scientific	14200-067	PBS is at 10X and should be diluted to 1X using ddH2O

			http://www.fishersci.com/ecomm/servlet/Search?keyWo
L-15 medium	Fisher Scientific	21083-027	Medium for atmospheres without CO2 control
Fibronectin	Sigma-Aldrich	F1141-5MG	http://www.sigmaaldrich.com/catalog/search?interface=A
Penicillin & Streptomycin	Fisher Scientific	15140-122	http://www.fishersci.com/ecomm/servlet/Search?keyWo
Petri dish P35	Greiner	627102	http://www.greinerbioone.com/en/row/articles/catalogu
Petri dish P60	Greiner	628163	http://www.greinerbioone.com/nl/belgium/articles/catal
Petri dish P94	Greiner	633179	http://www.greinerbioone.com/nl/belgium/articles/catal
			http://www.sigmaaldrich.com/catalog/product/sial/p614
Paraformaldehyde 3 %	Sigma-Aldrich	P6148-500G	Harmful in-particular for the eyes, working in a fumehood
Triton 0.5 %	Sigma-Aldrich	93443-100ML	http://www.sigmaaldrich.com/catalog/search?interface=A
Phallodin-Green Fluorescent Alexa			http://www.lifetechnologies.com/order/catalog/product/
Fluor 488	InVitrogen	A12379	dissolve powder in 1.5 ml methanol
Alexa Fluor 647	InVitrogen	A21245	1:200 dilution in PBS 1X
			1:500 dilution in PBS 1X
rabbit polyclonal anti-Giantin	Abcam	ab24586	http://www.abcam.com/giantin-antibody-ab24586.html
rabbit anti-anillin	Courtesy of M. Glotzer, Published in *Piek*		1:500 dilution in PBS 1X
Anti-phosphotyrosine	Transduction Lab	610000	http://www.bdbiosciences.com/ptProduct.jsp?ccn=61000
	Jackson		http://www.jacksonimmuno.com/catalog/catpages/fab-ra
Cy3 goat anti-rabbit	Immunoresearch	111-166-047	1:1000 dilution in PBS 1X
			http://www.sigmaaldrich.com/catalog/product/sigma/d8
DAPI	Sigma-Aldrich	D8417	1 mg/ml for 1 min
Glycerol	Sigma-Aldrich	G2025	http://www.sigmaaldrich.com/catalog/search?interface=A
Mineral oil	Sigma-Aldrich	M8410-500ML	http://www.sigmaaldrich.com/catalog/search?interface=A
HeLa cells	-	-	Mammalian cells are available from many companies. See
NIH3T3 cells	ATCC	-	Mammalian cells are available from many companies. See
Fission yeast	-	-	For details on strains, contact the corresponding author. S
C. elegans worms	-	-	For details, contact the corresponding author. See also Ta
YES (Agar) + 5 Supplements			http://www.mpbio.com/search.php?q=4101-732&s=Searc
included	MP Biomedicals	4101-732	For preparation: follow instructions as given on the box
YES (Media) + 5 Supplements			http://www.mpbio.com/search.php?q=4101-522&s=Searc
included	MP Biomedicals	4101-522	For preparation: follow the instructions as given on the bc
			http://www.mpbio.com/search.php?q=4110-012&s=Searc

EMM (Media)	MP Biomedicals	4110-012	For preparation: follow instructions as given on the box
Filter sterilized EMM (Media) - Only for imaging	MP Biomedicals	4110-012	For preparation: follow instructions as given on the box. F http://www.mpbio.com/search.php?q=4104-012&s=Searc
Supplements (for EMM)	MP Biomedicals	4104-012	(Add 225mg/l into the EMM media before autoclaving or
Stericup and Steritop Vaccum driven sterile filters	Millipore	-	http://www.millipore.com/cellbiology/flx4/cellculture_pr

ets and ensure a perfect surface flatness.

rom the manufacturer for more information.

rom the manufacturer for more information
?lang=en&region=CA

?lang=fr&region=FR
ta sheet from the manufacturer for more information.

36529?lang=fr&region=FR

is highly flammable (with ignition flashback able to occur across considerable distances), consequently it should be used in a fume cupbo
es/thin-mil-/57372-kleenguard-g10-blue-nitrile-gloves-m

s/index.jsp

.aspx?R=131EN
similar elastomers are available from multiple companies.

12301479&keyWord=41965-039&store=Scientific&nav=0&offSet=0&storeId=10652&langId=-1&fromSearchPage=1&searchType=PROD
056?lang=en&region=CA
rd=25200-072&store=Scientific&nav=0&offSet=0&storeId=10652&langId=-1&fromSearchPage=1&searchType=PROD
rd=14200-067&store=Scientific&nav=0&offSet=0&storeId=10652&langId=-1&fromSearchPage=1&searchType=PROD

rd=21083-027&store=Scientific&nav=0&offSet=0&storeId=10652&langId=-1&fromSearchPage=1&searchType=PROD

All&term=F1141-5MG&lang=en&region=CA&focus=product&N=0+220003048+219853082+219853286&mode=match%20partialmax
rd=15140-122&store=Scientific&nav=0&offSet=0&storeId=10652&langId=-1&fromSearchPage=1&searchType=CHEM
e/article/144_11/12885/
ogue/article/145_8_bl/24872/
ogue/article/146_8_bl/24882/
8?lang=fr&region=FR
is required; check the data sheet from the manufacturer for more information.
All&term=93443-100ML&lang=en&region=CA&focus=product&N=0+220003048+219853082+219853286&mode=match%20partialmax
'A12379?CID=search-a12379

)0
ab.asp

417?lang=fr&region=FR

All&term=G2025&lang=en&region=CA&focus=product&N=0+220003048+219853082+219853286&mode=match%20partialmax
All&term=M8410-500ML&lang=en&region=CA&focus=product&N=0+220003048+219853082+219853286&mode=match%20partialmax
also Table 1
also Table 1
See also Table 1
able 1
ch

ch
ox
ch

ilter sterilize the media using a 0.22 µm filter instead of autoclaving. This gives transparency to the media and reduces the autofluoresce
ch
filtering)

epare&tab1=2&tab2=1#tab2=1:tab1=2

ard away from sources of ignition

nce.

Table of Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: Wollrab_et_al_Equipment.xlsx

Name of Equipment	Company	Catalog Number
Spin-coater	Laurell Technologies corporation	WS-400B-6NPP/Lite
Oxygen plasma cleaner	Diener Electronic	Zepto B
Hot Plates	VWR International PI-KEM	- 1000-1 Precision hot plate
Mask aligner	SUSS MicroTec	MJB3
Nalgene Vacuum Dessicator	dominique Dutscher	029289
Vacuum pump	KNF	N022AN.18
Oven	Memmert	-
Custom-made coverslip holder for imaging	-	-
Custom-made cylinders	-	-
Centrifuge	eppendorf	5810R
Sonicator	Branson Ultrasonics	B200
Phase contrast microscope	Nikon	TS 100
Epifluorescence microscope	Nikon	Eclipse Ti
NIS Elements Imaging software (Ar)	Nikon	3.10, SP3, Hot fix (Build 644), LO.
CCD Camera	Photometrics Andor iQ	CoolSNAP HQ2 Andor iXon 897 BI
Confocal Microscopes	Leica TCS DMI 6000	Yokogawa CSU 22 spinning head SP5-MP SP8-MP

Spectrometer to measure optical density	Fisher Scientific	Cell density meter model 40.

Comments/Description

http://www.laurell.com/spin-coater/?model=WS-400-6NPP
http://www.plasma-us.com/649-0--zepto-low-cost.html
https://fr.vwr.com/app/search/Search?searchOp=OR&stiboGroup=590336
http://pi-kem.co.uk/hot-plates

http://www.suss.com/en.html

Use UV protection glasses

http://www.dutscher.com/frontoffice/home
http://www.knf.fr/dn_pompes_labo/
http://www.memmert.com

Custom-made; For details, contact the corresponding author.
Custom-made (height: 63mm/External radius: 26mm/Internal radius: 7mm); For more details see reference 11 (Allen T.D *et al*) in the main text.

http://online-shop.eppendorf.fr/Centrifugation/Centrifugeuses/Centrifuge-5810-5810-R-PF-18809.html

Available from many companies.
http://www.nikoninstruments.com/en_FR/Products/Microscope-Systems/Inverted-Microscopes/Eclipse-TS100-TS100F
http://www.nikoninstruments.com/en_EU/Products/Microscope-Systems/Inverted-Microscopes/Eclipse-Ti
http://www.nikoninstruments.com/en_EU/Products/Software/NIS-Elements-Ar-Microscope-Imaging-Software

http://www.photometrics.com/products/ccdcams/coolsnap_hq2.php

http://www.andor.com/scientific-cameras/ixon-emccd-camera-series

http://www.yokogawa.com/scanner/products/discontinued.htm

http://www.leica-microsystems.com/products/confocal-microscopes/details/product/leica-tcs-sp5-mp-2/

http://www.leica-microsystems.com/products/confocal-microscopes/leica-tcs-sp8-configurable-confocal/details/product/leica-tcs-sp8-mp/

https://webshop.fishersci.com/insight2_fr/getProduct.do?productCode=11899283

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Ordering cells and embryos in 3 dimensions: a new device for high content screening

Author(s): Viktoria Wollrab, David Caballero, Raghavan Thiagarajan, Daniel Riveline

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: [X] Standard Access [ ] Open Access

Item 2 (check one box):

[X] The Author is NOT a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	Daniel Riveline
Department:	Laboratory of Cell Physics ISIS/IGBMC
Institution:	University of Strasbourg and CNRS
Article Title:	Ordering cells and embryos in 3 dimensions: a new device for high content screening
Signature:	Daniel R.
Date:	2 Dec 2013

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

List of changes
Click here to download Rebuttal Comments: Wollrab_et_al_List of changes.docx

Editorial comments and *corresponding changes*:

1) Please be consistent with the font color throughout the manuscript.

Text changed to Calibri, 12pt, Black.

2) Please describe centrifuge speeds as "x g" instead of “g” or the machine-dependent "rpm". For instance in step 1.1.2, 1.1.10 etc.

We give the speed of rotation in g for all centrifuge processes, since this unit is independent from the used rotor. However, for spin coating processes the sample is always placed in the middle, so giving the speed in rpm is also machine-independent. Here the speed in g would be meaningless. So giving the speed in g for centrifuges and in rpm for spin coating, the reader can immediately apply these parameters without further calculations.

3) Please rephrase sentences that include “should be”, “can be”, “could be” etc. For example in step 1.1.3 you mention “the time shouls be adapted to..” rephrase this to “Adapt the time to..”. Please follow this comment for rest of the protocol step where applicable.

The proposed changes were implemented in the entire manuscript, concretely in 1.1.3, 1.1.4, 1.1.5(Note), 1.1.6(Caution), 1.1.8, 1.1.9, 1.2.6(Note), 1.3.1.1(Note), 1.3.1.7(Note), 1.3.2.10(Note), 2.4(Note).

4) In step 1.1.5 you mention “see NOTE” in parenthesis. However, below the step you mention two notes. Please consider removing “see NOTE” and combine both the notes below the step.

Notes were combined and ‘(see Note)’ removed.

5) Under step 1.1.6, please combine the Caution statements. And replaced “lamp should be checked” to “Check the power of the lamp..”.

Caution statements combined and sentence changed to “Check the power of the lamp..”.

6) Please make a note of “should be” and rephrase it in step 1.1.8 and 1.1.9. In caution statement consider rephrasing “let the wafer cool down” to “cool the wafer to..”.

In the steps 1.1.8/9 the "should be" sentence was rephrased and became a note. The caution statement was rephrased.

7) In step 1.1.12, please consider replacing "(Optional)" with "Alternatively,.." and include it in the sentence.

Changed to" This step is optional.".

8) Please replace the word " Sylgard 184, Dow Corning", "SigmaCoteTM" and "Falcon" with a more generic term as much as possible throughout the entire manuscript. JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Please remove all commercial sounding language from your manuscript. All commercial products should be sufficiently referenced in the table of materials/reagents.

'Falcon' was changed to 'tube', 'Sylgard 184, Dow Corning' and 'Kimtech' were removed, 'SigmaCoteTM' was replaced by 'siliconizing reagent', 'Parafilm' by 'plastic film' and 'Milli-Q' by 'ultrapure (corrections in 1.2.1, 1.2.2, 1.3.1.2, 1.3.2.5, 1.3.2.6, 2.5, 2.6, 4.2.1, 4.2.4, 4.2.6, 5.1.4, 5.2.3, 5.2.3(Note), Fig. 2 caption, Figure 2, Table1). These corrections were also applied in the table of materials.

9) Please check for grammar in "note" under step 1.2.1.

Rephrased to "Using a 1:10 (v/v) ratio is also working".

10) In the note under step 1.2.3, please consider rephrasing to " If air bubbles appear during this step, degas..".

Rephrased as proposed.

11) Under step 1.2.5 note please replace "can vary" with "varies" and consider beginning "This time will.." as a new statement.

"Varies" and new sentence implemented as proposed. Similar change in 5.1.7(Note).

12) Please revise the protocol text to avoid the use of any pronouns (i.e. "we", "you", "your", "our" etc.). If you feel it is very important to give a personal example, you may use the royal "we" sparingly and only

as a "NOTE:" after the relevant protocol step. For example under step 1.3 “We next describe..”. Please consider rephrasing it to “In this step..”. For your convenience, please use the Ctrl+F function to search for individual pronouns and edit them.

We checked the manuscript as proposed with the ctrl-F function and careful proof reading. The following steps were rephrased correspondingly: 1.2.5(Note), 1.3, 1.3.1.8, 2.7(Note), 3, 3.1(Note), 3.5(Note), 4.2.8(Note), 4.3, 4.3.1(Note), 5.1, 5.1.4(Note), 1.1.6(Note and Caution), 4.1.1(Caution).

13) Following step 1.3.1.7, you mention 3 separate “NOTES”. We encourage authors to use the “NOTE” function sparingly. Please consider including any of the notes into the protocol and re-number it appropriately.

The first note is rephrased. The second note is included in the step 1.3.1.7. The last note is rephrased and included as step in the protocol. Same change in the note below step 1.3.2.3. Now it is step 1.3.2.4 and the subsequent steps were re-numbered.

14) Please ensure your protocol section in imperative tense, as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.).

Change in 1.3.1.1(Note), 1.3.1.3(Note), 1.3.1.7(Note), 1.3.2.1(Note), 2.1(Note), 2.5(Note), 5.1.4(Note), 5.1.5, 5.2.3(Note).

15) After you have made all of the recommended changes to your protocol (listed above), please re-evaluate the length of your protocol section. There is no page limit for the protocol text, but there is a 3 pages limit for filmable content. If your protocol is longer than 3 pages, please highlight 2.75 pages (or less) of text to identify which portions of the protocol are most important to include in the video; i.e. which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVEs instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

The length was reevaluated and the highlighted parts are not longer than 2.75 pages. So no changes.

16) Please move the “Tables and Figures” section above “Discussion” below “Representative Results”. Similarly, please move “Disclosures” after “Acknowledgments”.

Implemented as proposed.

17) Please make sure your discussion section to includes all the following points.

a) Critical steps within the protocol.

b) Modifications and troubleshooting.

c) Limitations of the technique.

d) Significance of the technique with respect to existing/alternative methods.

e) Future applications or directions after mastering this technique.

Some sentences were added to the discussion, to elaborate the points a) to e) further.

Additional:

The 'µ'-sign was not the same everywhere. This is unified now. (change in step 1.3.1.2, 1.3.1.3, 2.2, 2.3, Table1).

'Spin coat' was replaced by 'spin-coat' in step 1.1.2, 1.3.1.3(Note), 1.3.2.3, 1.3.2.7, 'Equipment' table.

'Centrifuge' was replaced by 'Spin-coat' in step 1.3.1.3.

'Petri dishe' was replaced by 'Petri dish' in the 'Materials' table.

'eggcups' changed to ' 'eggcups' ' to unify (step 2.10(Caution), 3.3, Figure1 Caption).

Answers to referees and list of changes
Click here to download Rebuttal Comments: Reply_to_Referees.docx

Strasbourg, April 9th 2014

Dear Dr. Jaydev Upponi,

Thank you for your email and for the comments. We answer below and we have corrected the manuscript accordingly.

Could we schedule already the acquisition for the movie in Strasbourg ? Second half of june would be ideal for us.

With best regards,

Daniel Riveline

CC: elizabeth.sheeley@jove.com

Dear Dr. Riveline,

Your manuscript JoVE51880R1 'Ordering cells and embryos in 3 dimensions: a new device for high content screening' has been peer-reviewed and the following comments need to be addressed.

Please keep JoVE's formatting requirements and the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

Please use the "track-changes" function in Microsoft Word or change the text color to identify all of your manuscript edits. When you have revised your submission, please also upload a list of changes, where you respond to each of the comments individually, in a separate document at the same time as you submit your revised manuscript.

Editorial comments:

The editor made the following changes to the manuscript.

1.The editor moved "Check the power.." to the beginning of step 1.1.6.

2.The editor changed "Adapt the time.." to "Optimize the time..".

3.The editor combined step 1.1.9 with 1.1.8 now, 1.1.8. Additionally, the editor removed the "Caution" sign below step 1.1.8 and combined "After the post-baking, cool the wafer.." with step 1.1.8.
4.The editor renumbered step 1.1.9 and 1.1.10.

5.The editor un-highlighted the heading "Strategy 1".

6.In step 1.3.1.7, the editor replaced "Note" with "Caution!".

7.The editor removed the "Caution" sign and combined the statement with step 1.3.2.7.

8.The editor added "of fibronectin solution" in step 2.3

9.The editor combined the step 2.9 and 2.10, now step 2.9

10.The editor mentioned "see step 4.1" in step 2.16.

11.The editor combine step 3.1 and 3.2, now 3.1 and adjusted the numbering of the steps below.

12.In the paragraph below step 3, the editor removed all the commercial names such as Nikon and Leica. Similarly below step 5.

13.The editor highlighted step 3.1 for better continuity.

14.In step 4.1, the editor combined very small steps such that each step has 2-3 action items.

15.The editor combined step 5.1.2 and 5.1.3, now, 5.1.2. Similarly, 5.1.6 , 5.1.7 and 5.1.8, now 5.1.6. Similarly, from 5.2.4-5.2.8, now step 5.2.4.

16.Keeping the length of the highlighted section in mind and over all continuity, the editor un-highlighted the steps below protocol step 5

Answer: We implemented all these changes and we agree with these modifications.

Please retain all the above changes and please address the following comments.

1) Please take this opportunity to thoroughly proofread your manuscript to ensure that there are no spelling or grammar issues. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

Answer: The new version of the manuscript was read carefully and additionally the following changes were made:

1. The words 'coined', 'glass' of 'glass coverslip', 'and' of 'Cavities were covered with fibronectin to facilitate adhesion and cells were inserted by centrifugation.', 'and' of 'Filling percentage was optimized for each system allowing up to 80% and cells and

embryos viability was confirmed.' were deleted and 'for the study of active processes' was replaced by 'to study active processes'. All changes were done to reduce the length of the abstract to 300 words.

2. The word 'release' was replaced by 'Remove' in step 4.1.1.
3. The sentence in Page 10, Line 331: 'Alternatively an upright microscope was used,' was changed to 'Alternatively an upright confocal microscope was used'.
4. In agreement with the editorial comment no 12, the commercial names in page 13, section 4.3 were removed and the paragraph was changed to: 'For this example an upright confocal microscope is used, equipped with PMT and Hybrid detectors. A 25X or 63X HCX IR APO L water objective (0.95 NA,) was selected to provide a wide field of the sample and show the applicability of the device for high-content-screening applications'. Similarly, the commercial names in page 14, section 5.2, line 464 were removed and the sentence was changed to: 'The observation was performed using an inverted phase-contrast microscope equipped with a 40X air objective 0.55 NA.

5. The sentence in Page 13, section 5.1, line 420 was changed to: 'For fission yeast observation an inverted spinning disk confocal microscope was used.'

2) Please keep in mind that we do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

Answer: In this work, we show representative results that demonstrate the strength of our method. Our claims are supported by data and by published references.

Reviewers' comments:

Reviewer #1:

Title:

"Ordering cells and embryos in 3 dimensions: a new device for high content screening" The title is confusing. It can be expected that they present a method to ordering cells in 3 dimensions. From the manuscript, they show a method to ordering cells in a 2D array. The point is that each individual cell is located in an individual 3D well.

This should be clarified in the title and the others parts of the manuscript.

Answer: We agree with the reviewer and we changed the title for: *''Ordering single cells and single embryos in 3D confinement: a new device for high content screening''*. The addition of the word 'single' highlights the fact that individual cells/embryos are located in an individual 3D well with orientation of the ring for example. We also took into account the comment of Reviewer #2 (see below).

Short abstract:

The authors claim "Their 3D confinement mimics physiological conditions". In my opinion the 3D wells allows a 3D environment for the cells. This is an important point to study but the 3D wells do not "mimics physiological conditions" as stated by the authors. Among others, cell mechanotransduction or cell-cell communication can also play an important role.

Answer: We agree that the statement can be formulated more specifically. We changed the sentence for: "*Their 3D confinement is a step towards 3D environments encountered in physiological conditions*". Page 2, Lines 47-49.

Abstract:

The claim "This device consists of an array of microcavities" It would be better to change to "This device consists of a 2D array of microcavities".

Answer: The sentence was changed accordingly. Page 2, Lines 61

Protocol:

Page 4 "Place the wafer with the photoresist layer with circular features of 20 μm (see Note) in diameter into contact with the photolithographic mask on the mask aligner" This sentence is confusing.

Answer: The sentence was modified for: ´Load the wafer on the mask aligner for UV exposure. Place the photolithography mask on it. The mask shows a pattern of circular features (disks) of 20 μm in diameter´. Page 4, Lines 136-138 in step 1.1.5.

Page 4 "…NOTE: Diameters of disks should.." define disks.

Answer: We define disks: 'Dimensions of disks dimensions on the mask will determine the diameter of cavities in the device'. Page 5, Lines 144-145, below step 1.1.5.

Page 13 "These conical 'eggcups' can be fabricated by means of Deep Reactive Ion Etching technology in a specialized microfabrication center." Explain better how to produce the conical eggcups

Answer: This is a standard microfabrication procedure which, for the sake of simplicity, we prefer not to detail the protocol in length and prefer to refer to the published patent and references therein, on Page 13, Line 433 (ref. 13) in the Note of step 5.1.1.

Representative Results.

The authors claim "The 'eggcups' are a novel high-content-screening methodology which allows the visualization of oriented cells and embryos in a 3D physiological environment". It would better to change "3D physiological environment" by "3D environment". In the physiological environment the mechanical properties of the surrounding environment of the cells can also play a role.

Answer: We changed the sentence along the suggestion of the referee, on Page 15, Line 483.

Reviewer #2:

Manuscript Summary:

Review: JoVE51880R1 - Ordering cells and embryos in 3 dimensions: a new device for high content screening, Wollrab et. al.

The authors describe a method for the production of micro-well arrays for confinement of cells and embryos for screening applications. The manuscript is well presented and describes the method of production of the micro-wells and the subsequent usage for screening experiments in a way suitable for a researcher in the field to replicate the experiment, providing a useful technique for both single cell/embryo analysis and population studies. The work completed is accurate and complies with research standards for cell analysis. The manuscript would benefit from the addition of more background on some of the techniques used (e.g. photolithography and soft lithography) and an examination of competing technologies.

Answer: We thank the reviewer for these comments. We added some elements along this line of suggestions (see below).

Major Concerns:

Points of note:

** I am not sure that the title accurately reflects the work completed. "Ordering of the cells and embryos in 3 dimensions: …" suggests a fully 3-D ordering of the cells where cells are arranged in the z- direction as well as the x- and y-. In this work, the cells are ordered in a 2-D array (x- and y- only), although individual cells are confined in a way that more closely resembles the 3-D physiology of the cell in the body, compared to 2-D surface culture. As such, I would suggest that the authors reconsider the title to more accurately reflect the work. My suggestion would be something along the lines of "Ordered micro-well arrays for 3-dimensional confinement of cells and embryos: a new device for high content screening".*

Answer: We agree with the reviewer, although the suggested title does not reflect the proper orientation of the cytokinetic ring for example. We propose: ´Ordering single cells and single embryos in 3D confinement: a new device for high content screening´.

** In the abstract, the authors refer to the "identification of features" using the micro-wells. The authors should expand on this to give an example(s) so that someone reading the abstract has an indication of these features.*

Answer: We changed the sentence for: "This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure and compacted phenotypes for Golgi and nucleus alignment". We have included this on Page 3, Lines 70-71 .

** In the short abstract, the authors describe the work as the production of a "new" device for cell culture. Others have previously reported on the production of micro-wells for cell culture (e.g. reference 11) therefore I would suggest the authors define the novelty of their devices, or remove the "new".*

Answer: We changed the sentence for: '*a device and a new method'*. The novelty in orientation is detailed in the next sentence of the short abstract on Page 2, Short abstract.

** A small discussion on the choice of the dimensions for the micro-wells would be helpful. Is there a way of defining well sizes that accommodate the cells? The authors state that the wells can be "adapted" (line 625), but how much adaptation is required, and how much can the micro-wells be designed for different cell types/embryos?*

Answer: We added the following sentences. ´The dimensions of ´eggcups´ were selected in the following manner: dimensions of cells which undergo division were measured on 2D surfaces: they have a spherical shape and their diameter was taken as a good indication for the EC diameter. Cells in ´eggcups´ elongate and orient along their long axis during cell division for example. This dimension depends on the system - cells and embryos - so this dimension should be evaluated in each case.' We have implemented this on Page 15, Lines 490-496.

** In a similar vein, if the well sizes are not a good fit for the dimensions of the cell, what effects does this have on the subsequent culture experiments? Does a "badly fitting" well start to approximate to a 2-D surface rather than a 3-D confinement? How can the culture experiment compensate for dimensional irregularity? If the cell does not "fit" into the micro-well, there will still be some scope for "stress". Is it that there has to be some compromise between stress and conformity?*

Answer: If well diameters are too small, cells/embryos do not enter. If they are too large, the orientation of organelles will not be observed. The challenge and novelty consist in matching the cell/embryo dimensions with the micro-well diameter.

** I cannot find Trimethylchlorosilane (TMCS) in the materials list. The authors should include a warning about the use of TMCS in the text. TMCS produces acute inhalation and dermal toxicity, and is highly flammable (with ignition flashback able to occur across considerable distances), consequently it should be used in a fume cupboard away from sources of ignition.*

Answer: These elements are added in the new version of the materials list. We have also included a warning about the use of TMCS in the main manuscript on Page 7, Lines 211-213..

* It is my understanding that the production of the PDMS depends on the manufacturers recipe, and that different recipes give PDMS with different properties (elasticity, surface hydrophobicity etc.). The properties of the PDMS are not discussed in the manuscript, but may be relevant for the later functionalization and culture studies. Can the authors comment on the properties of the PDMS used here, and the variety of PDMS types available, and refer the reader to the manufacturer's specifications in each case.

Answer: The PDMS commonly used comes from Dow Corning. To our knowledge, the product is pretty robust, and most of the laboratories currently working in this field (*e.g.* G.W. Whitesides, M. Piel, M. Thery, B. Ladoux…) usually mention the same standard protocol.

** Section 1.3.1: How do the authors ensure any residual TMCS silane does not affect the subsequent steps of the functionalization and culture?*

Answer: We thank the reviewer for this observation. We performed extensive washing steps in order to ensure that the functionalization of the PDMS stamp with TMCS did not affect the subsequent fibronectin adhesion and cell culture on the EC. The obtained results supported this; cell behavior was not affected, and in particular, division kinetics was the same as compared to standard 2D cultures. Additionally, we did not observe any difference between *Strategy 1* (using TMCS) and *Strategy 2* (using Sigmacote™, another siliconizing agent) (see section 1.3.2). Both strategies, using different products to replicate the ´eggcups´, lead to the very same results. Altogether, cells are not affected by the treatment with TMCS. We have though, added a comment about cleaning the PDMS stamp on section 1.3.1.7 (Page 7, Line 233) and 1.3.2.10 (Page 8, Line 270).

Minor Concerns:

Minor points:

** The use of the term "egg-cups" is somewhat redundant as the field already has a term - micro-wells - which adequately describes the structures produced.*

Answer: We understand, but the word ´eggcups´ capture the novelty: matching the short axis of an ellipse to orient vertically individual cells/embryos. We would like to keep it.

** Can the authors refer to some literature work to give readers some background on the use and versatility of photolithography and soft lithography/hot embossing, especially in their use with respect biomedical applications? This will allow the reader access to more in-depth examinations of the respective fabrication techniques, underpinning the experiments described here.*

Answer: We now quote two new references (Ref. 15 and 16) in the Introduction section, on Page 43, Line 108:

1) Mehling and Tay, 2014, Curr Op Biotech **25**: 95-102.
2) Wolfe *et al*. 2010, Rapid Prototyping of Microstructures by Soft Lithography for Biotechnology, Microengineering in Biotechnology, Methods in Molecular Biology Volume 583, 2010, pp 81-107

** Can the authors give examples of some similar cell confinement systems already on the market, e.g. for small area/population cell culture or analysis*

Answer: We now quote micro-wells from other Companies on Page 20, Lines 702-703.

** The immobilisation of individual cells in a large array is extremely useful for the examination of populations of cells and their response to external stimuli. Can the authors give examples of literature population experiments that may utilise the micro-well arrays.*

Answer: We now quote the following papers at the end of the Introduction section, on Page 4, Lines119-121:

'Finally, our device will be useful for studying the distributions of cells responses to external stimuli, for example in cancer (Yao, X. et al. Integ Biol **6**, 388-398 (2014)) or in basic research (Eberwine et al., Nat Meth **11**, 25-27 (2014)).'

** Section 1.2.6: Does the PDMS need to be peeled off the substrate before cutting out, or should it be cut out and then peeled off?*

Answer: It should be cut first. This has been clarified on the note of section 1.2.6 on Page 6, Line 196.

** Section 1.3.1.2: Can the authors define "upside"; is it the side with the structures on?*

Answer: We define it on Page 6, Line 205 as ´the side with the structures´.

** Lines 712 and 722: Should "training" be replaced with "practice"?*

Answer: We agree with the referee. We have modified it accordingly, on Page 19, Line 656 and 657..

** Line 151: should "appropriated" be replaced with "appropriate"?*

Answer: We agree with the referee. We have modified it accordingly, on Page 5, Line 156.

Additional Comments to Authors:
N/A

Reviewer #3:

Manuscript Summary:

The paper reports a method to study cells and embryos in microcavities. The idea behind is to provide cells with a 3D microenvironment and by this mimic better the physiological culture conditions. The fabrication method is thoroughly explained and applied to a few examples: study of organelles and the closure of the cytokinetic ring during cell mitosis.

Answer: We thank the reviewer for the comments and answer below.

Major Concerns:

Although the paper deals with a very interesting topic my major concerns raise from its main focus. If it is a paper focused in the fabrication of the device then I am missing the added value of this device with other alternatives already found in literature such as the ones published by Kwon et at, Analytical Chemistry 2011 ir Khetani et al, Nature biotechnology 2008 to just cite some of them.

Answer: The added values are connected to the 3D environment for single cells and embryos, and the orientation of cellular/embryos organelles compared to the quoted references.

I think authors should comment more on the benefits of their approach (no need of clean room maybe? simplicity?) and comment more on their limitations.

Answer: These points are now added in the MS. *'Our approach is simple and does not need a clean room*'. Page 4, Line 117 and Page 19, Lines 645-646.

For instance, for how long can they have the cells in culture in such device?

Answer: Cells can be cultured until confluence with this device. But the main interest is when individual cells/embryos are isolated within the cavities. This is specified on Page 20, Lines 675-677.

In small cavities liquid handling and in particular medium exchange can be a problem and will for sure limit the kind of experiments that can be addressed using this platform.

Answer: Since our device is 'open' above cavities, we checked that medium exchange was not a problem. Cells in EC did not show any degradation for both short and long-term experiments due to lack of medium exchange. In particular, cell cycle was not altered. This was added on Page 20, Lines 674-675..

On the other hand, to apply this device in drug screening, how will be the controls defined?

Answer: The controls will be defined as the mean signal/phenotypes of the read-out in the absence of drugs.

Would that be in other (parallel) device?

Answer: This could be in the same device in a multi-well plate for example. Since the MS is already long, we prefer to leave these issues for future developments of the method. However, we comment on the future developments of our assay on Page 20, Lines 697 and 698.

As a lot of the process is performed manually, how the variability would affect control /sample in such cases?

Answer: The device will be developed for industrial (drug screening) applications at the bottom of a multiwell plate. As such, high content screening assays will be performed following the current automatised processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability. This point is added on Page Page 20, Lines 697-699.

Regarding the protocol itself I find some details missing. One important issue is sterility, how is that performed in such devices?

at which point of the protocol?

Answer: Sterility of EC is ensured by UV exposure. This has been clarified on Page 9, Line 280, in section 2.3. Note also that a caution note about sterility was included on Page 9, Line 286 below section 2.5.

The other issue is the time that cell should be into the microcavities before rinsing to remove unattached cells, I could not find it.

Answer: Cells are 'captured' by the cavities either by their shapes (for fission yeast) or by adhesion molecules (for mammalian cells). The rinsing occurs immediately after centrifugation. This is described (and clarified) in section 2.13 on Page 10, Line 317-319.

On the contrary, if the main focus of the paper is on the new applications of such device I find that the novelties should be properly remarked and discussed and this should be done starting with the abstract. In this they claim "This device allowed the identification of new features which were unknown by using standard 2D cell culture assays" but they do not give specific information to make such features relevant.

Answer: We took the option to present the novelty of the approach with illustrations. As suggested by the referee, we have included the novelties in the abstract: ´This device allowed the identification of new features, such as periodic accumulations and in-homogeneities of myosin and actin during the cytokinetic ring closure, compacted phenotypes for Golgi and nucleus alignment´. These changes have been implemented on Page 3, Lines 70-71.

As the closure of the cytokinetic ring during cell mitosis has been already observed in similar devices by the same authors they should provide more information about what the new findings are.

Answer: As agreed with the editor - this paper was an invitation by JoVE - this paper will appear after the publication of the main results. In this work (JoVE), we provide new findings about the different phenotypes of the Golgi apparatus and the study of the nucleus orientation. In addition, we give more information about the cytokinetic ring and the capabilities of the EC for being applied in different model systems (mammalian cells, yeast, C.*elegans*…) which has not been shown so far.

Moreover, new applications of the device would probably need the mimicking of cell-cell and cell-ECM contacts. They just briefly menction this in the discussion and I would have loved to see how they envisage to include such modifications into the device.

Answer: We have further developed this point: ´In this setup single cells are captured. This is in contrast to epithelial tissues encountered *in vivo*. However, this environment could be reproduced in our 'eggcups' by coating the side walls with cadherins to mimic cell-cell contacts using more flexible elastomers. Focal contacts will be promoted by the deposition of fibronectin at the bottom of the wells. These respective distributions of adhesion molecules should allow in reproducing the cellular environments encountered *in vivo*. By this method one would approach the physiological conditions.´

We have implemented this change on Page 19-20, Lines 668-674.

Finally, high throughput techniques in the end should include some automatization in the fabrication processes but also in the visualization techniques and this may also be included or considered in this device.

Answer: We agree with the referee. We now discuss on Page 20, Lines 698 and 699 the applicability of the ´eggcups´ for high-content-screening applications, in particular for industrial-oriented purposes:

´As such, high-content-screening assays will be performed using the commonly used automatised processes of pharmaceutical companies (and academic research laboratories) using robots. This will ensure repeatability and reliability with low variability.´

Minor Concerns:
N/A

Additional Comments to Authors:
N/A

Your revision is due by **Apr 09, 2014**.

To submit a revision, go to the *JoVE* submission site and log in as an author. You will see a menu item called 'Submission Needing Revision'. You will find your submission record there.

Sincerely,

Jaydev Upponi, Ph.D.
Science Editor
JoVE
1 Alewife Center, Suite 200, Cambridge, MA 02140
tel: 617-674-1888