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Automated microinjection of cell-polymer suspensions in 3D ECM scaffolds for high-throughput quantitative cancer invasion screens

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ABSTRACT

Cell spheroids (CS) embedded in 3D extracellular matrix (ECM) serve as *in vitro* mimics for multicellular structures *in vivo*. Such cultures, started either from spontaneous cell aggregates or single cells dispersed in a gel are time consuming, applicable to restricted cell types only, prone to high variation, and do not allow CS formation with defined spatial distribution required for high-throughput imaging. Here, we describe a method where cell-polymer suspensions are microinjected as droplets into collagen gels and CS formation occurs within hours for a broad range of cell types. We have automated this method to produce CS arrays in fixed patterns with defined x-y-z spatial coordinates in 96 well plates and applied automated imaging and image analysis algorithms. Low intra- and inter-well variation of initial CS size and CS expansion indicates excellent reproducibility. Distinct cell migration patterns, including cohesive strand-like – and individual cell migration can be visualized and manipulated. A proof-of-principle chemical screen is performed identifying compounds that affect cancer cell invasion/migration. Finally, we demonstrate applicability to freshly isolated mouse breast and human sarcoma biopsy material – indicating potential for development of personalized cancer treatment strategies.

1. Introduction

Cells grown under classical 2D culture conditions behave differently from the same cell types grown *in vivo*. In addition to soluble factors produced in the *in vivo* microenvironment, differences in cell shape, intercellular contacts, and connections to ECM have striking effects on gene expression, cell survival, proliferation, differentiation, cytoarchitecture, and migration. Various systems have been developed to culture cells within 3D ECM environments, aimed at more closely mimicking the *in vivo* context [1,2]. Several of these systems produce 3D cell aggregates in which, after compaction, depletion of oxygen, nutrients, and growth factors occurs in the core, leading to cell heterogeneity depending on the position in the resulting cell spheroids (CS) [3,4]. Multistep methods are used in which aggregates are allowed to form spontaneously and, following a compaction phase, can subsequently be transferred to a 3D ECM. The best-known example of this approach is the "hanging drop assay" that was developed to create embryoid bodies from ES cells and has also been applied to cancer cell lines to produce tumor-like structures [5,6]. Alternative methods involve mixing of single cell suspensions with a solidifying ECM, resulting in individual cells that eventually form CS randomly within a 3D ECM structure [7], or seeding polymeric scaffolds with cell/ECM suspensions [2].

Cell behavior in 3D cultures is controlled by chemical (composition) and physical (rigidity, cross-linking) properties of the gel. Natural ECM proteins can be used such as collagen, fibrinogen, or the laminin-rich matrigel to represent the *in vivo* ECM composition most relevant to a given cell type. More recently, synthetic polymers have been developed for 3D CS culture environments although it remains to be established how well these support a variety of cell behavioral outputs, including cell migration [8]. Collagen type 1 is an abundant polymer in ECM *in vivo*, and it is widely used for 3D cultures. Various physical properties of the collagen gel, such as rigidity and pore size modulate stem cell differentiation, cancer growth, and cell migration [9–11]. Cells can use various migration strategies in 3D environments, including mesenchymal or amoeboid individual cell migration modes or collective invasion strategies,

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depending on properties of the cells and of the matrix [10]. Changes in matrix pore size can force cells to adopt alternative migration strategies or – if too extreme – pose a barrier to cell migration. Importantly, cells can modify the ECM by physical deformation and proteolysis, to overcome such barriers [12].

Chemical compound screens as well as RNAi screens for various types of cellular functions, including survival, growth, differentiation, and migration are mostly performed in 2D culture conditions. Methods to analyze cells in 3D based on the hanging-drop assay are labor and time intensive; are limited to cell types that are cohesive and aggregate spontaneously; and are prone to high variability between experiments due to variation in aggregation and compaction time and CS size. Alternative methods in which single cell suspensions are mixed with soluble ECM substrates that are subsequently allowed to form a gel are relatively easy to perform but also have several major disadvantages: formation of CS depends on the ability of a cell type to survive and proliferate as single cells in low adhesion conditions for extended periods; CS formation is time consuming; CS show a large variation in size; and CS form at random locations, which is disadvantageous for imaging purposes.

To allow for CS formation that is relatively fast and easy, highly reproducible, and overcomes the disadvantages described above we have developed a method where cell-polymer suspensions are microinjected into multiwell plates containing a collagen gel. This method has been automated to produce CS arrays with highly reproducible properties in large quantities in 96 well plates. We use this system to visualize distinct 3D migration strategies and regulation of those strategies by ECM properties and actomyosin contractility. We demonstrate applicability in high-throughput screening platforms in a chemical screen for compounds that affect breast cancer invasion/migration. Finally, we apply the method to cell suspensions derived from fresh tumor biopsies, which opens the possibility to test therapeutic strategies on freshly isolated material from individual patients.

2. Materials and methods

2.1. Cell culture

The following cell lines were obtained from ATCC: MDA-MB-231, MTLn3, PC-3, HT1080, 4T1, and MAE. GEβ1 was described earlier [13]. All cell lines were cultured under standard cell culture conditions indicated by ATCC or as described [13] at 37 °C, 5% CO_2 in a humidified incubator. Primary mouse tumor cell suspensions were derived from surplus mouse breast tumor material by mincing using scalpel and tissue chopper followed by 2-h collagenase treatment at 37 °C. Human biopsy material was obtained from surplus material from patients that were surgically treated for chondrosarcoma or osteosarcoma. Tumor cell suspensions were derived from biopsies by 12 h collagenase treatment at 37 °C. All human specimens were handled in an anonymized coded fashion according to the National ethical guidelines for secondary use of patient-derived material.

2.2. Preparation of collagen

Collagen type I solution was obtained from Upstate-Milipore or isolated from rat-tail collagen by acid extraction as described previously [14]. Collagen was diluted to indicated working concentrations of ~2.4 mg/ml in PBS containing 1xDMEM (stock 10x, Gibco), 44 mM $NaHCO_3$ (stock 440 mM, Merck), 0.1 M Hepes (stock 1M, BioSolve).

2.3. Hanging drop method

~5,000 cells in 20 μl droplets were dispensed onto a 10 cm dish that was inverted over a dish containing 10 ml DMEM. After 24 h, cell aggregates were harvested using a Pasteur pipette and transferred into 10 cm dishes coated with 0.75% agarose submerged in 10 ml DMEM. After 48 h CS had formed and these were embedded into a 2.4 mg/ml collagen solution using a Pasteur pipette. Collagen gels were allowed to solidify at 37 °C for 30 min and overlaid with DMEM. Cell invasion was recorded for 3 days using an inverted phase contrast light microscope (Nikon Eclipse E600).

2.4. Cell preparation for injection method

Cell suspensions derived from trypsin-detached adherent cultures or from collagenase-treated biopsies were filtered to remove clumps, centrifuged at 1000 rpm for 5 min, and washed twice with PBS. ~7×10^6 cells were re-suspended in 30 μl PBS containing 2% polyvinylpyrrolidone (PVP; Sigma–Aldrich). The PVP/cell suspension was loaded into a beveled pulled glass needle (Eppendorf CustomTip Type III, oD [μm] 60, Front surface 40, Flexibility: rigid).

2.5. Manual injection

Cell suspensions in 2% PVP were microinjected (~1×10^4 cells/droplet) with a microinjector (20 psi, PV820 Pneumatic PicoPump, World Precision Instruments, Inc) into solidified collagen gels in 8 well μslides (IBIDI).

2.6. Automated injections

A glass-bottom 96 well plate (Greiner) containing 60 μl solidified 2.4 mg/ml collagen gel per well was placed in a motorized stage (MTmot 200 × 100 MR, Märzhäuser) connected to a controller (Tango, Märzhäuser). A motorized micromanipulator (Injectman II, Eppendorf) was positioned above the stage and connected to a pump (Femtojet Express, Eppendorf) featuring an external compressor (lubricated compressor, model 3–4, JUN-AIR). A firewire camera (DFK41BF02.H, The Imaging Source) equipped with an 8× macro lens (MR8/O, The Imaging Source) was placed beneath the stage for calibration and imaging. All components were connected to the controlling computer (Ubuntu AMD64). A multi-threaded control program was written in Python using PySerial and wxPython. Coriander software (http://damien.douxchamps.net/ieee1394/coriander) was used for imaging.

After the program was calibrated for the 96 well plate the camera height was adjusted to focus on the bottom of the 96 well plate. The plate was then removed for needle calibration: the injection needle was fixed in the Injectman and moved, using the Injectman controller, into the center of the image. The injection height was set to 200 μm above the bottom of the (virtual) plate. After the needle was moved up, the plate was placed back in position and the upper left well was used for multiple test injections to adjust pump pressure and injection time for optimization of the droplet size (~8 nl ≈ 300 μm diameter) using video inspection. Subsequently, using a predefined macro defining x-y coordinates and number of injections per well, all wells were injected with the same pressure and injection time.

2.7. Microscopy and image analysis

Manually injected CS were monitored daily using a Nikon Eclipse E600 microscope. CS generated by automated injection were used for montage imaging using a Nikon TE2000 confocal microscope equipped with a Prior stage controlled by NIS Element Software and a temperature and CO_2-controlled incubator.

Differential interference contrast (DIC) images were captured using a charged coupled device (CCD) camera with NIS software at 10× dry objective. Quantification of CS invasion area was analyzed from DIC images using ImageJ. The CS ellipsoidal area after three days was estimated using the diameter in *x* and *y* axis (pi*radius-x*radius-y) occupied by cells in the 10× montage image in the mid-plane of each CS and normalizing to the occupied area 1 h after injection. One-way ANOVA was performed to test the significance of the data. The data are presented and plotted as average and standard error of the mean.

For automated imaging, wells containing gel-embedded CS were treated with a fixation and staining cocktail containing 3.7% paraformaldehyde, 0.2% Triton X-100 (Sigma) and 0.1 μM rhodamine Phalloidin (Sigma) for 3 h. Wells were washed extensively with PBS and plates were imaged on a Becton Dickinson Pathway 855 using a 4× lens. A montage of 12 frames was made for each Z plane, with a total of 24Z planes at an interval of 50 μm. Image stacks were converted into 2D maximum fluorescence intensity projections using ImagePro 7.0. CS were then digitally segmented using ImageJ to identify the outline of individual CS and multiple parameters were measured, including Feret's diameter, roundness, and number of CS scored in each well.

For immunostaining of E-cadherin, gels were incubated for 30 min with 5 μg/ml collagenase (Clostridium histolyticum, Boehringer Mannheim) at room temperature, fixed with 4% paraformaldehyde, permeabilized in 0.2% Triton X-100, and blocked with 10% FBS. Gels were incubated with E-cadherin antibody (BD Transduction Laboratories) overnight at 4 °C followed by Alexa 488-conjugated secondary antibody (Molecular Probes/Invitrogen) for 2 h at room temperature and Hoechst 33258 nuclear staining (Molecular Probes/Invitrogen) for 30 min at room temperature. Preparations were mounted in Aqua-Poly/Mount solution (Polysciences, Inc) and analyzed using a Nikon TE2000 confocal microscope. Z-stacks (~100 stacks, step of 1 μm) were obtained using a 20× dry objective, imported into ImageJ, and collapsed using extended depth of field plugin (Z projection) into a focused composite image.

2.8. Drug treatment

LY-294002 (phosphatidylinositol 3-kinase), JSI-124-cucurbitacin (STAT3/Jak2), NSC23766 (Rac1), and AG-82 (general protein tyrosine kinases) were purchased