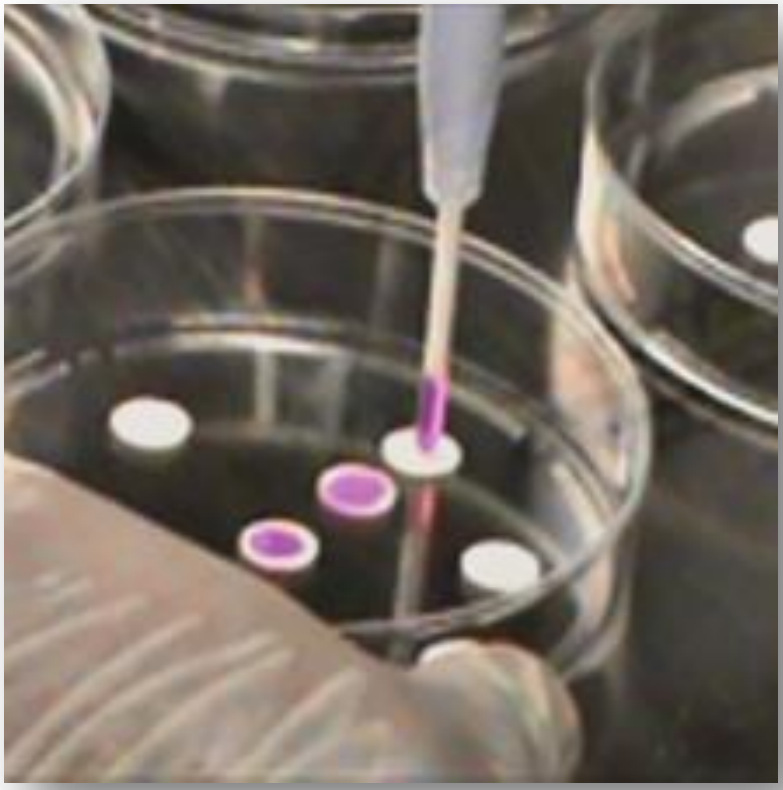
LENA BIOSCIENCES
3D cell culture tools for life sciences www.lenabio.com

SEEDEZ™ PROTOCOLS

USER GUIDELINES AND PROTOCOLS FOR THE SEEDING OF THREE-DIMENSIONAL CELL CULTURES



INTO THE SEEDEZ™

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Plating of 3D Cell Cultures Into the SEEDEZ™

December 2018

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INTRODUCTION

Cells seeded into the SEEDEZ™ may be of various origins and sources. Cells may be primary cells, secondary cells and cell lines. Please continue to use protocols normally used for cell maintenance prior to seeding. If cell lines or secondary cells are used, maintain cells in flasks until they are ready to be seeded into the SEEDEZ™.

USER GUIDELINES AND RECOMMENDATIONS

To prepare cells for seeding, continue with protocols used for plating cells. This may include cell dissociation from a flask, followed by centrifuging and the re-suspension of cell pellet in a medium in which cells will be seeded into the SEEDEZ™. Count live and dead cells using automatic counters, or hemocytometer and Trypan Blue exclusion.

Depending on the equipment available, seed cells into the SEEDEZ™ using micropipettes, automated pipettes, dip-in or wick-in method as shown in Lena Biosciences video tutorials.

Please visit www.lenabio.com for more information.

MIXED CELL CULTURES

To seed mixed cell cultures into the SEEDEZ™, resuspend each cell type separately and then join them together prior to seeding. Always have in mind what are desired total live cell density and the ratio of heterogeneous cell types at seeding.

ADHERENT CELLS

To seed adherent cell types, pre-coat SEEDEZ™ with cell adhesive molecules and follow protocols used for the coating of cell ware disposables. You may also seed cells in a diluted or undiluted sol-state extracellular matrix (ECM) or hydrogel into uncoated SEEDEZ™, or you may pre-coat SEEDEZ™ and then seed cells in undiluted or diluted ECM or hydrogel.

CELLS IN AN EXTRACELLULAR MATRIX OR GEL

If you previously worked with 3D gel-based cell cultures or extracellular matrix barriers, continue with protocols used for cell seeding in a gel you commonly use. The following are the most common seeding methods:

A. Cell seeding in a sol-state gel suspension.
B. The seeding of invasive cells on top of an extracellular matrix barrier for invasion assays.
C. Cell seeding in a "sandwich" between, for example, a layer of Collagen I and a layer of Matrigel.

SEEDEZ™ allows for cell seeding using all three approaches.

CELL SEEDING IN A SOL-STATE GEL SUSPENSION

Cells may be seeded in a sol-state gel suspension; or SEEDEZ™ may be coated with cell adhesive ligands or extracellular matrix (ECM) constituents, followed by addition of a cell suspension in a sol-state gel or ECM.

SEEDING OF INVASIVE CELLS ON TOP OF AN ECM BARRIER FOR INVASION ASSAYS OR OTHER USES

If the objective is to seed invasive cells on top of the extracellular matrix (ECM) barrier, dispense sol-state ECM into the SEEDEZ™ first, let the ECM gel and then add cells. In this approach cells sit on top of the ECM at the start of experiment.

You may also seed cells in a sol-state ECM suspension into the SEEDEZ™. In contrast with previous approach, this provides for truly 3D cell migration/invasion assay, representative of *in vivo* conditions, in which cells are embedded in the extracellular matrix (ECM) from the assay start to its end.

CELL SEEDING IN A "SANDWICH" BETWEEN TWO LAYERS OF EXTRACELLULAR MATRIX

If you previously used "sandwich" cultures, you may have had only one cell layer between two layers of extracellular matrix at plating. If SEEDEZ™ is used, 3D cell culture will be truly 3D at seeding, comprising multiple layers of cells embedded in a 3D matrix. To achieve this, you may first coat the SEEDEZ™ with chosen molecules (Collagen I, for example) and then dispense cells in a sol-state ECM suspension such as Matrigel. Alternatively, you may use two SEEDEZ™ substrates comprising gelled extracellular matrix to seed cells in a sandwich between the substrates, or you may seed cells in the third SEEDEZ™ substrate sandwiched by two SEEDEZ™ substrates.

THREE-DIMENSIONAL FEEDER LAYER

SEEDEZ™ is a versatile tool which allows to plate different cell types at a user convenience and in a desired sequence. For example, you may seed one cell type first and then add a different cell type. Different cell types may also be seeded at the same time and one cell type may be a feeder cell. This is important for culturing of difficult to culture cell types, while still providing for metabolic support by way of feeder cells which are typically cultured in 2D. Feeder cells may be, for example, astrocytes supporting neurons or inactivated fibroblasts metabolically supporting stem cells.

The SEEDEZ™ is unique; it enables to add three-dimensionality even to a feeder cell layer while culturing or co-culturing other cells in 3D. To provide for 3D-distributed feeder cells in a co-culture, or to provide for conditioned medium for another 3D culture of dissociated cells or cell aggregates, one of the following approaches may be used:

A. Non-contact co-culture method

   Coat a SEEDEZ™ substrate, if needed, and seed feeder cells. Coat another SEEDEZ™ substrate, if needed, and seed fastidious cells or cell aggregates. Place the first SEEDEZ™ substrate in a culture well. Place the second SEEDEZ™ substrate in a well insert in the same well at a desired point in time.

B. Contact co-culture method

   Coat the SEEDEZ™, if needed, and seed feeder cells. Next, seed fastidious cells or cell aggregates at a time you choose. Alternatively, coat SEEDEZ™, and seed fastidious cells / cell aggregates together with feeder cells.

   For contact co-culturing in a single SEEDEZ™ substrate with the feeder cells seeded first, followed by seeding of fastidious cells, you may spot cultures side-by-side or spot-a-second-culture on top of the first culture spot. THE SEEDEZ™ SUBSTRATE SHOULD NOT BE SATURATED WHEN YOU ARE ADDING THE SECOND CULTURE SPOT. Alternatively, feeder cells may be seeded in one SEEDEZ™ substrate and fastidious cells in another SEEDEZ™ substrate and then overlapped.

C. Conditioned medium

Coat a SEEDEZ™ substrate, if needed, and seed feeder cells. Coat another SEEDEZ™ substrate, if needed, and seed fastidious cells or cell aggregates. Keep the substrates in separate wells. Add conditioned medium to the well seating the second substrate using an amount of medium from the well seating the first substrate.

If you wish to keep feeder layers planar, you may still do so. First, plate the feeder layer. Next, coat the SEEDEZ™ substrate if needed, and seed difficult to culture cell types. At a desired time, place the SEEDEZ™ substrate over the planar feeder layer (contact co-culture) or place it in an insert in the same well (non-contact co-culture).

STACK-AND-CULTURE

3D cell cultures embedded in different SEEDEZ™ substrates may be overlapped. To do this, simply place one or more SEEDEZ™ substrates on top of each other. You may overlap cultures to the extent you wish, and you may overlap them at any time.

IF CULTURES ARE NOT BROUGHT TOGETHER AT THE TIME OF CELL SEEDING AND THE OBJECTIVE IS TO STUDY CELL MIGRATION, INVASION, CHEMO-INVASION OR ANGIOGENESIS, YOU MAY NEED TO ADD A LAYER OF EXTRACELLULAR MATRIX BETWEEN THE SEEDEZ™ SUBSTRATES TO PROVIDE FOR GOOD SUBSTRATE-TO-SUBSTRATE ADHESION.

SEEDEZ™-SANDWICH CULTURES

You may seed cells in a sol-state gel between two SEEDEZ™ substrates; the two SEEDEZ™ substrates may contain gel and/or cells. Alternatively, seed cells in a SEEDEZ™ substrate such that the SEEDEZ™ is sandwiched between two layers of gel, a layer of gel and another SEEDEZ™ substrate, or between two SEEDEZ™ substrates.

MULTILAYERED TISSUE MODELS, SEEDEZ™-STACK

You may use a stack of SEEDEZ™ substrates to model tissues normally comprising multiple layers; a neocortex, or cerebral cortex for example. Each tissue layer typically comprises layer-specific cell types and multiple layers of cells all of which may be mimicked by a culture in the SEEDEZ™ substrate.

Use one SEEDEZ™ substrate to model a tissue layer. Remember this is a tissue layer, not a layer of cells, which you may mimic by a 3D cell culture in the SEEDEZ™. Next, place the SEEDEZ™ substrates on top of each other in the order in which the tissue layers are organized in the tissue. You may also add "tissue layers" using cells or cells in an extracellular matrix seeded between the SEEDEZ™ substrate(s), or above and/or below the SEEDEZ™ substrate(s), as necessary.

SIDE-BY-SIDE CULTURE

Side-by-side culturing refers to a method of co-culturing in which a user may spot a culture, followed by spotting of another cell culture in the same SEEDEZ™ substrate. This allows to add one or more cell sub-populations to a cell population, or to seed different cell populations for the purpose of modeling tissue heterogeneity in health or disease; for example, normal, neoplastic and tumor cell mass. Cell populations and sub-populations may include cells from various origins and sources and may be from donors of various ages and/or stages of disease progression. Cells may be normal cells, diseased cells, cancer cells, stem cells etc.

TO ACHIEVE THE MOST CONSISTENT AND REPRODUCIBLE RESULTS, ALL CELL POPULATIONS AND SUB-POPULATIONS SHOULD BE SEEDED FIIRST AND THEN MEDIUM ADDED.

MIGRATION, INVASION, CHEMO-INVASION AND ANGIOGENESIS ASSAYS

SEEDEZ™ allows you to tailor cell migration, invasion, chemo-invasion and angiogenesis assays to your experimental goals and research objectives. It also provides for greater experimental flexibility; flexibility which cannot be achieved using a fixed insert, with a fixed membrane, and with a fixed extracellular matrix barrier. While this is not the most extensive list of all SEEDEZ™ uses in this context, SEEDEZ™ may be used to enable or provide one or more of the following:

A. Control of extracellular matrix (ECM) composition or gel concentration in one or more SEEDEZ™ substrates.

B. Addition and embedding of a test compound into the SEEDEZ™.

C. Addition of a test compound to a sol-state ECM followed by embedding into the SEEDEZ™.

   Test compounds may be matrix metalloproteinase inhibitors, angiogenic inhibitors, actin polymerization inhibitors, chemokines, chemoattractants, or other modulators of cell motility, or another cell function.

D. Seeding of different cell types into the same or different SEEDEZ™ substrates with the objective of joining them; for example, the seeding of invasive cells in one substrate and the seeding of normal cells in another substrate with or without extracellular matrix.

E. Seeding of cells + ECM + test compound into the SEEDEZ™.

F. Addition of extracellular matrix on top or below the SEEDEZ™ substrate(s).

G. "Spotting" of different cultures or spotting of different cell types in the same SEEDEZ™ substrate.

   For example, spot invasive cells and spot non-invasive cells in an extracellular matrix into a SEEDEZ™ substrate.

H. Spot-a-Culture™ and Spot-a-Drug™ in the same or different SEEDEZ™ substrate.

I. Development of custom assays by overlapping SEEDEZ™ substrates.

IF THE OBJECTIVE IS TO ALLOW CELLS FROM ONE SEEDEZ™ SUBSTRATE TO INVADE OR MIGRATE INTO THE ABOVE OR BELOW SEEDEZ™ SUBSTRATE, THEN THE ADHESIVE LAYER BETWEEN THE SEEDEZ™ SUBSTRATES SHOULD BE A LAYER OF EXTRACELLULAR MATRIX USED IN ONE OR BOTH SUBSTRATES.

IF THE OBJECTIVE IS TO ALLOW A TEST COMPOUND FROM ONE SEEDEZ™ SUBSTRATE TO DIFFUSE INTO ANOTHER SEEDEZ™ SUBSTRATE CONTAINING CELLS IN A HYDROGEL, THEN REDUCE OR OMIT ADDITION OF CULTURE MEDIUM TO THE WELL DURING SHORT-TERM DRUG DIFFUSION STUDIES IN A HUMIDIFIED INCUBATOR.

SEEDING METHODS AND OPTIMAL DISPENSING VOLUME

You may use three seeding approaches to embed any cells in any sol state suspension into the SEEDEZ™:

A. Spot-a-Culture™

B. Wick-a-Culture™

C. Dip-and-Culture™

SPOT-A-CULTURE

You may spot cultures using any micropipette available. However, when dispensing cells in a sol-state gel, and viscous or otherwise difficult to dispense reagents, you may want to consider using a positive displacement micropipette to reduce losses in pipetting and to obtain the most consistent results.

The following examples show how proteinaceous Matrigel® extracellular matrix spots look like when dispensed using both a positive displacement micropipette (see Example 1) and a standard laboratory micropipette (see Example 2). Lena Biosciences video tutorials also teach how easy it is to plate consistent cultures even when these cultures are dispensed in a Matrigel extracellular matrix sol-state suspension.

EXAMPLE 1: MATRIGEL SPOTS DISPENSED USING A POSITIVE DISPLACEMENT MICROPIPETTE

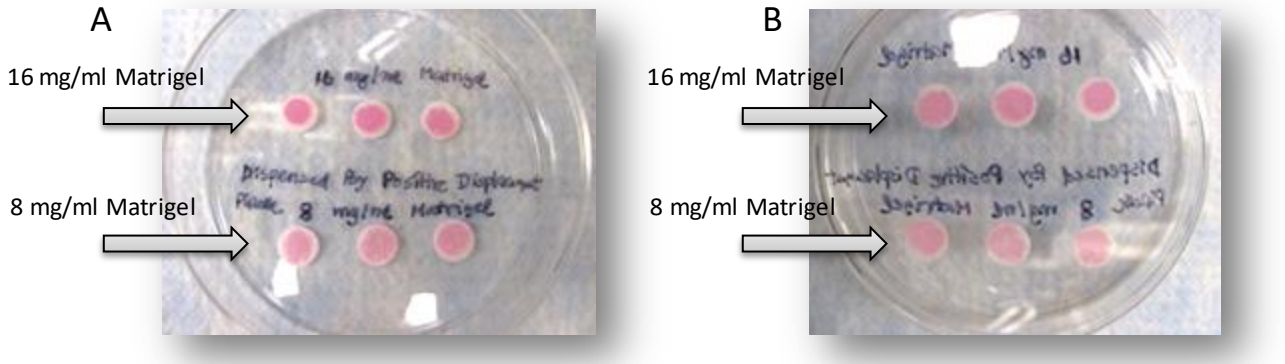


Fig. 1 Growth factor reduced (GFR) Matrigel at a final concentration of 16 mg/ml and 8 mg/ml dispensed using a hand-held positive displacement micropipette (Gilson Microman).
A Front view of the samples. **B** Back view of the samples.

Properly chilled Matrigel cell suspension is difficult to plate consistently in 3D and in high-throughput onto standard cell ware disposables. Typically, cultures spread during pipetting and transfer to incubator before Matrigel gels. Certain coatings such as Poly-D-Lysine make Matrigel cell cultures spread even more. Example 1 and Example 2 demonstrate that consistent Matrigel cultures can be formed with ease using SEEDEZ™ even without automated dispensing equipment. Formed cell cultures are three-dimensional, consistent, reproducible, and suitable for vigorous handling, as they are wicked by and self-contained in respective SEEDEZ™ substrates.

Matrigel® is an extracellular matrix comprising structural proteins such as Laminin, Collagen IV, and Entactin which present cultured cells with adhesive peptide sequences they often encounter in their natural environment. When using Matrigel®, a common laboratory procedure is to dispense small volumes of chilled (4 °C) Matrigel onto plastic tissue culture labware, or SEEDEZ™, using frozen pipette tips. This is because Matrigel is a thermo-reversible hydrogel which is in sol state at 4 °C, and gels at higher temperatures. If Matrigel is not chilled enough, then it may start gelling before it fully permeates the SEEDEZ™ substrate (see Fig. 1A, the first substrate in the first row); however, even in such state, the viscous solution permeated the SEEDEZ™. In contrast, without the SEEDEZ™, the shape and characteristic dimensions of 3D cell culture is difficult to reproduce culture-to-culture if plated onto standard labware under this condition.

EXAMPLE 2: MATRIGEL SPOTS DISPENSED USING A STANDARD LABORATORY MICROPIPETTE

A

B

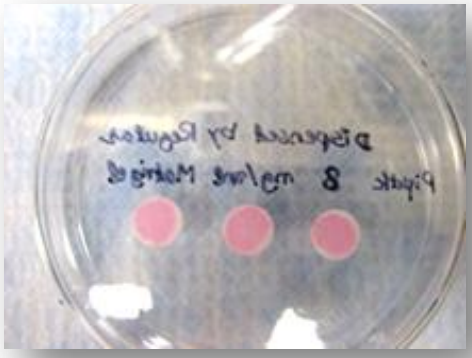


Fig. 2 Growth factor reduced (GFR) Matrigel at a final concentration of 8 mg/ml dispensed using a standard laboratory micropipette and standard pipette tips.

A Front view of the samples. **B** Back view of the samples.

For a fixed dispensing volume, the reproducibility and the spread of culture spots, gel spots, or drug spots in the SEEDEZ™ depend on chemical and physical properties of dispensed reagent, and its sensitivity to environmental parameters and their temporal and spatial variations, among other factors. For the most consistent results, please examine the specification sheet and the application requirements for the reagent you are dispensing into the SEEDEZ™. For Matrigel, manufacturer recommends use of frozen pipette tips and cell ware disposables to prevent sol-state ECM gel from gelling prior to and during dispensing. As can be seen from the Example 1 and Example 2, properly chilled Matrigel (4 °C) yields reproducible spots even when dispensed using standard laboratory micropipette and standard pipet tips, if delivered into the SEEDEZ™, and even when only pipet tips and not the SEEDEZ™ were pre-chilled.

Batch-to-batch variations in the composition of a reagent you wish to spot may also contribute to spot variability. While SEEDEZ™ makes these differences less apparent and produces geometrically more consistent cultures, the cultures will not be identical at seeding if the composition of a suspension in which cells are seeded is changed. For example, culture spread may be greater or smaller than what you found in the previous reagent batch for otherwise identical conditions at seeding. If Matrigel is used, slight variations in composition may be present batch to batch. To investigate this possibility, Growth Factor Reduced Matrigel from two different lots was tested, as shown in the Example 3. However, significant differences in Matrigel spread, *if dispensed into the SEEDEZ™* were not observed.

For a given volume of dispensed solution, factors which yield the greatest variations in culture dimensions (in x, y and z) and cell distribution across these dimensions at seeding are:

A. Losses in pipetting.
B. Relative change in position of the pipet tip from the center of the substrate. *

*In the Examples 1-3, Matrigel was dispensed manually with the pipet tip touching approximately the center of the SEEDEZ™ substrates.

EXAMPLE 3: SPREAD OF HIGH PROTEIN MATRIGEL FROM TWO DIFFERENT LOTS

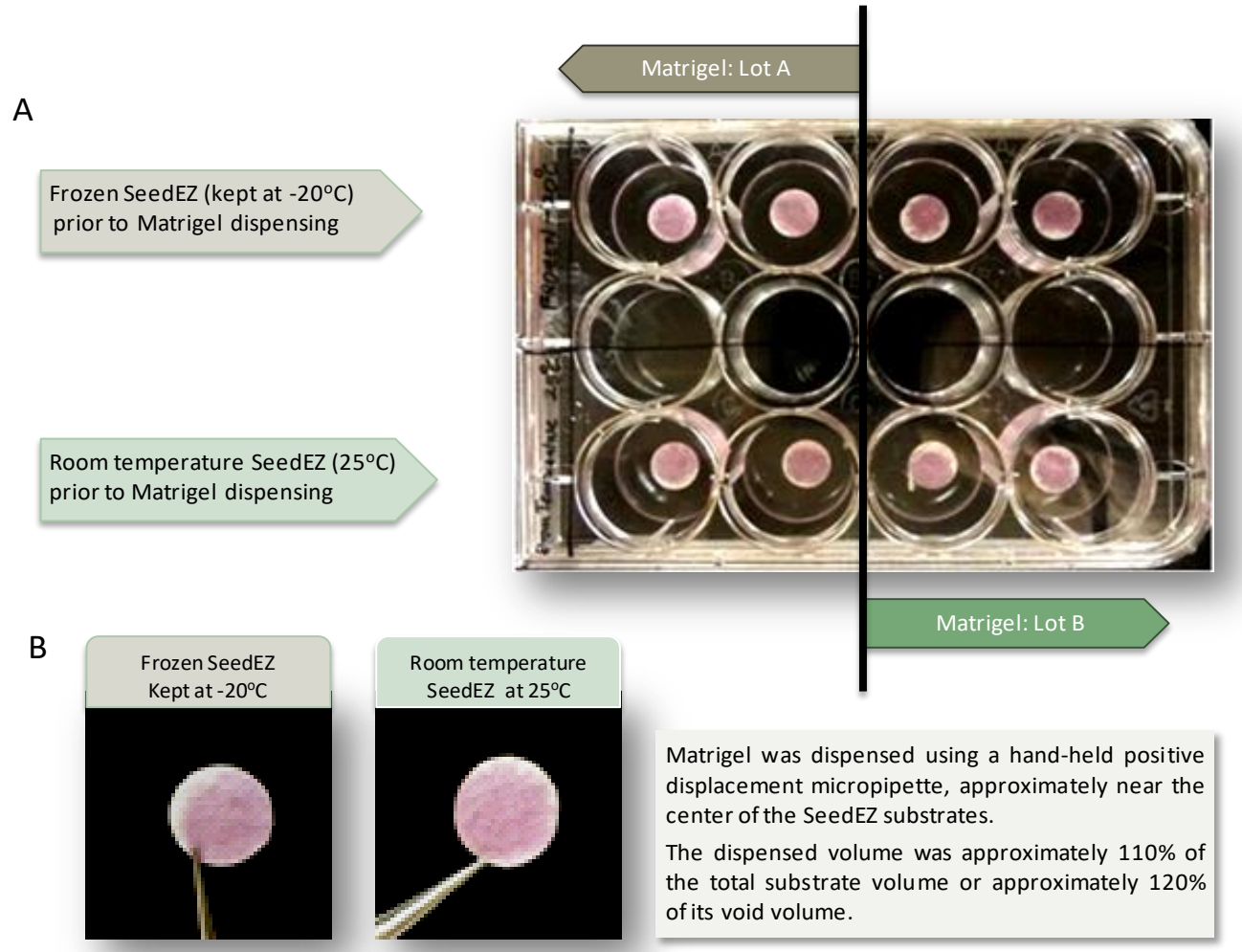


Fig. 3 Reproducibility of Matrigel spread in the SeedEZ substrates.
A. The spread of high protein concentration, 16 mg/ml GFR Matrigel into the SeedEZ as a function of Matrigel lot and the SeedEZ substrate temperature.
B. Close-up view of the SeedEZ substrates showing Matrigel spread from Lot A as a function of the SeedEZ substrate temperature prior to Matrigel plating.

3D CELL CULTURES WILL BE MORE CONSISTENT, THAT IS, THE CULTURES WILL BE OF MORE CONSISTENT SHAPE, SPREAD, THICKNESS AND CELL DISTRIBUTION IN X, Y AND Z DIMENSIONS, CULTURE-TO-CULTURE AND BATCH-TO-BATCH IF YOU USE SEEDEZ™ FOR ALL YOUR 3D CELL CULTURE NEEDS.

OPTIMAL DISPENSING VOLUME FOR SPOT CULTURES

You may spot any volume of cells in any sol-state suspension. However, perfectly circular culture spots are obtained only when the dispensed volume is approximately twice the void volume of the SEEDEZ™ substrate. For spot cultures plated into the SEEDEZ™ SC-C048 substrate this volume is approximately 50 µl.

An optimal dispensing volume is the volume that meets both of the following conditions:

1. A volume necessary to entirely wet and saturate the SEEDEZ™ substrate.
2. A volume which is after dispensing self-contained within the SEEDEZ™ substrate.

The first condition provides for a 3D cell culture in which cells are distributed *throughout* the SEEDEZ™ substrate. The second condition saves cells and reagents by minimizing the amount of cell suspension that leaves the SEEDEZ™ substrates if the substrate was over-saturated by it.

LENA BIOSCIENCES MAKES NO WARRANTIES THAT 50 µL IS AN OPTIMAL DISPENSING VOLUME FOR SC-C048 SEEDEZ™ SUBSTRATE. THIS VALUE DEPENDS ON CHEMICAL AND PHYSICAL PROPERTIES OF THE REAGENT, MICROPIPETTE USED AND LOSSES IN PIPETTING, THE SEEDEZ™ COATING (IF ANY), THE SURFACE TREATMENT AND COATING (IF ANY) OF THE CELLWARE IN WHICH THE SEEDEZ™ IS PLACED, AMONG OTHER FACTORS.

YOU MAY NEED LOWER OR HIGHER DISPENSING VOLUME THAN 50 µL TO CONSISTENTLY SATURATE SEEDEZ™ SC-C048 SUCH THAT DISPENSED VOLUME IS SELF-CONTAINED BY THE SUBSTRATE. HOWEVER, IT IS RECOMMEND THAT 50 µL BE USED AS A STARTING POINT FOR SC-C048.

HOW TO DETERMINE OPTIMAL DISPENSING VOLUME FOR YOUR APPLICATION?

To find optimal dispensing volume for your application, we recommend the following procedure:

1. Dispense 50 µl of reagent you will be using at cell seeding in each of the six SC-C048 SEEDEZ™ replicates. Do so in the same environment in which you will be plating cells, using the same micropipette, cell disposable in which SEEDEZ™ will be placed, and using the same coating applied to SEEDEZ™ prior to cell seeding, if any.
2. If 50 µl saturates SC-C048 SEEDEZ™ substrate but results in excess liquid surrounding the substrate, reduce dispensing volume to 40 µl and test it with six SEEDEZ™ replicates.
3. If 40 µl saturates the substrates and you cannot see excess liquid when you raise the SEEDEZ™ substrate using tweezers, then use 40 µl as your dispensing volume.
4. Repeat step 3 using cell suspension. You may need to adjust dispensing volume one more time.

The procedure may need to be repeated more than twice to ensure consistency or to further reduce dispensing volume by repeating steps 1-4 in 5 µl increments.

If the substrates are not fully saturated with 50 µl in step 1, increase dispensing volume to 60 µl in step 2. If the substrates are still not saturated, repeat steps 1-3 by increasing dispensing volume by 10 µl each time until the substrates are fully saturated, or until you see excess liquid. You may need to repeat this procedure more than twice to ensure consistency or to optimize dispensing volume in 5 µl increments.

ALTERNATIVE SOLUTION FOR DIFFICULT TO DISPENSE REAGENTS: IF YOUR REAGENT IS DIFFICULT TO DISPENSE WITHOUT SIGNIFICANT LOSSES IN PIPETTING, USE WICK-IN OR DIP-IN METHOD. THE SEEDEZ™ WICKS MOST SOL-STATE GELS AND CELL CULTURE REAGENTS, AND PRODUCES CONSISTENT CULTURES, GEL SHAPES, OR DOSE FORMS IN HIGH-THROUGHPUT WITHOUT EXPENSIVE EQUIPMENT OR COMPLEX PROTOCOLS.

WHAT TO EXPECT?

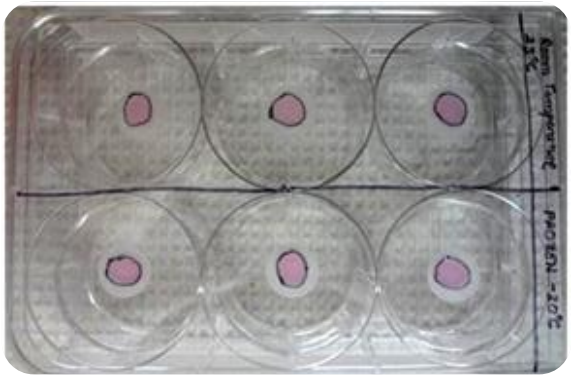
For the majority of cell suspensions and other reagents normally used in cell culture applications, dispensing volume less than the optimal volume produces an elliptically shaped, rather than a circularly shaped spot. However, the shape of the spot is reasonably consistent across the SEEDEZ™ substrates so long as the

dispensed volume is constant and losses in pipetting negligible; this makes 3D cultures and side-by-side 3D cultures consistent even if they are not perfectly circular.

As can be seen in the Example 4, the shape of 25 µl spot is elliptical, but consistent (dispensing was done manually without precise control over the position of the pipet tip relative to that of the center of the substrate). However, the closer the dispensed volume to the optimal dispensed volume, the more circular the spot becomes.

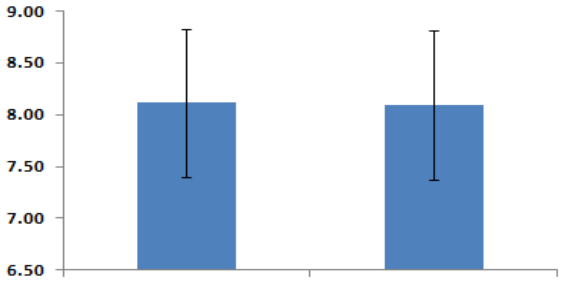
EXAMPLE 4: THE SPREAD OF 25 µL MATRIGEL™ SPOTS IN SC-C048 SEEDEZ™ SUBSTRATE

A



B Measured spread of 25 µl of 16 mg/ml Matrigel

Vertical axis: The average diameter of spread footprint in mm



C

25 µl of high-protein concentration GFR Matrigel was dispensed using a hand-held positive displacement micropipette, approximately near the center of SeedEZ substrates.

Matrigel spread was measured using calipers with three measurements per spread. For both sample conditions, the Matrigel spread in SeedEZ SC-C048 was statistically indistinguishable at diameters of 8.11 ± 0.71 mm and 8.09 ± 0.72 mm, for the SeedEZ samples kept at 25°C and -20°C, respectively, prior to gel plating.

Fig. 4 The spread of Matrigel spots for sub-optimal dispensing volume as a function of the SC-C048 temperature.

A. 16 mg/ml Matrigel spots for sub-optimal (25 µl) dispensing volume.
B. Measured Matrigel spread as a function of the SeedEZ substrate temperature.
C. Close-up view of elliptically shaped air-dried Matrigel spots.

WICK-A-CULTURE/ DIP-AND-CULTURE

If you are making a large number of 3D cell cultures using dissociated cells, and you do not have automated equipment or a multichannel pipette, use the wick-in or dip-in seeding method.

Wick-a-culture refers to a cell seeding method in which the SEEDEZ™ is placed in contact with the sol-state cell suspension, which the SEEDEZ™ wicks until it is saturated as shown in Fig. 5A. Dip-in seeding method is analogous to the wick-in method with the exception that the SEEDEZ™ substrate is fully immersed into the cell suspension.

Regardless of whether the SEEDEZ™ was partly or completely immersed into the cell suspension, for rapid seeding of many substrates you may dip them all at once; in a reagent reservoir, for example. This seeding

approach makes reproducible and consistent 3D cell cultures (Figs. 5B-5C) easier and faster, and without expensive equipment or complex protocols.

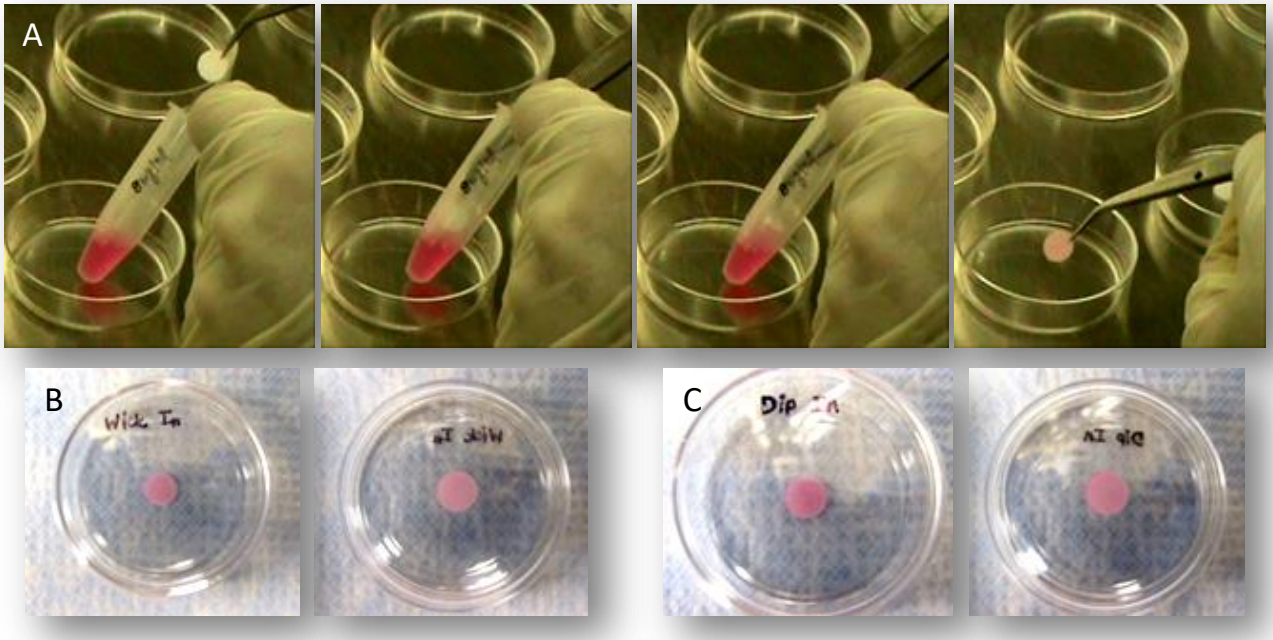


Fig. 5 Wick-a-Culture and Dip-and-Culture method.

A. Wick-a-Culture seeding method using a centrifuge tube and tweezers.
B. Front and back view of the SeedEZ substrate following Wick-a-Culture seeding method.
C. Front and back view of the SeedEZ substrate following Dip-and-Culture seeding method.

! REGARDLESS OF THE SEEDING METHOD USED, AFTER SEEDING, PLEASE TRANSFER CULTURES EMBEDDED IN THE SEEDEZ™ TO 5% CO_2 37°C INCUBATOR AND ALLOW AT LEAST 10 MINUTES AND UP TO 30 MINUTES OR LONGER FOR CELLS TO ADHERE TO THE INTERIOR OF THE SEEDEZ™ BEFORE ADDING MEDIUM.

The actual time it takes for cells to adhere depends on the cell type, whether the SEEDEZ™ was saturated with cell suspension at seeding, the SEEDEZ™ coating, whether the cells were seeded in medium or a sol-state gel suspension, the application and research objectives.

PROTOCOL: The seeding of 3D mixed cultures into Poly-D-Lysine coated SEEDEZ™

This protocol describes how to seed mixed 3D cell cultures of brain cells into a Poly-D-Lysine coated SEEDEZ™ SC-C048 in a 48-well plate. * The mixed population of cells comprised primary cortical neurons, and secondary (1X passaged) astrocytes and microglia. At seeding, the cell ratio was 2:1 neuron:glia at a total live cell density of 4 x 10^6 cells/ml.

** Courtesy of Dr. Laplaca's Lab, Coulter Department of Biomedical Engineering, Georgia Institute of Technology*

THE PROTOCOL DOES NOT PROVIDE DETAILS OF E-18 PRIMARY CORTICAL NEURON ISOLATION AND DISSOCIATION, AND P0-P1 ASTROCYTE/MIXED GLIA ISOLATION. THESE PROTOCOLS ARE AVAILABLE AT:

http://www.ncbi.nlm.nih.gov/pubmed/17409489

Cullen DK, Vukasinovic J, Glezer A, Laplaca MC.

Microfluidic engineered high cell density three-dimensional neural cultures.

J Neural Eng. 2007, 4(2):159-72.

ALTERNATIVE CELL SOURCES:

Cells, cortices, and whole brains may be obtained from vendors such as BrainBits. E-18 cortices may also be used as glial cell source. For ordered cells/ tissues, please follow isolation and maintenance protocols provided by the cell/ tissue supplier.

POLY-D-LYSINE (PDL) COATING

MATERIALS

- Poly-D-lysine Hydrobromide MW >300 kDa lyophilized powder, γ-irradiated; Sigma-Aldrich Product No. P7405
- Sterile, de-ionized water
- Disposables: SEEDEZ™ substrates SC-C048, a 48-well plate, conical tubes, and micropipette tips

METHODS

1. Prepare 100 µg/ml Poly-D-Lysine coating solution in sterile DI water. Store at 2-8 °C.
2. Place SC-C048 SEEDEZ™ substrates into a 48-well plate inside the hood.
3. Add 250 µl of 100 µg/ml Poly-D-Lysine solution to each well comprising SEEDEZ™.
4. Cover the plate by lid and transfer to incubator for at least 6 hours. Overnight incubation is recommended.
5. Transfer the plate to hood and aspirate PDL solution.
6. Rinse the substrates 2X with sterile DI water.
7. After you aspirate the last rinse, dry the SEEDEZ™ substrate by placing a pipet tip on the substrate and aspirate any remaining liquid from the SEEDEZ™. You will notice that SEEDEZ™ changes color from transparent to white when dry.

8. Cover the plate. PDL-coated SEEDEZ™ substrates are ready for use.

MAINTENANCE AND DISSOCIATION OF P0-HARVESTED MIXED GLIAL CELLS PRIOR TO SEEDING

MATERIALS

- Dulbecco's Modified Eagle Medium: Nutrient mixture F-12 (DMEM/F-12)
- Fetal Bovine Serum (FBS)
- Phosphate-Buffered Saline Ca^{++} and Mg^{++} free (PBS) 1X
- 0.25%Trypsin-EDTA
- Neurobasal Medium
- B-27 Supplement
- G-5 Supplement
- GlutaMAX
- HBSS, Trypan Blue and hemocytometer for cell counting
- Disposables: T-75 flasks, conical tubes, serological pipettes, and micropipette tips

METHODS

1. Dissociate two neonatal cortices and plate into a T-75 flask in 10 ml of DMEM/F-12 + 10% FBS.
2. Feed cells every 2-3 days thereafter, and passage or dissociate for seeding as follows:
3. Transfer 0.25% Trypsin-EDTA (1X) and medium to a 37°C water bath to warm up.
4. Aspirate medium from the flask.
5. Add 10 ml of 1X PBS to the flask and gently rinse the cells. Aspirate PBS.
6. Add 3 ml of 0.25% Trypsin-EDTA (1X) and gently tilt the flask to ensure that the entire bottom surface is covered.
7. Transfer flask to incubator for 3-5 minutes.
8. Gently tap the side of the flask to detach cells. Observe cells under the microscope:

   If most cells are detached, transfer the flask to hood.

   If cells are still adhered, place the flask back to incubator for 1 minute; repeat this step until cells are detached.
9. Add 7 ml of medium (DMEM/F-12 + 10% FBS) to the flask to neutralize Trypsin.
10. Use 10 ml serological pipette and rinse the bottom of the flask with medium/Trypsin mixture repeatedly to collect as many cells as possible.

11. Transfer cell suspension to a 50 ml conical tube and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).

12. Remove supernatant and re-suspend cell pellet.

13. Count cells using hemocytometer and Trypan Blue exclusion.

14. Add seeding medium to have total glia live cell density of 2.67 x 10^6 cells/ml in seeding medium (Neurobasal + 2% B-27 + 1% G-5 + 0.5 mM GlutaMAX).

SEEDING

MATERIALS

- Cells: neurons, astrocytes and microglia
- Medium: Neurobasal, B-27 Supplement, G-5 Supplement, GlutaMAX
- PDL-coated SEEDEZ™ SC-C048 in a 48-well plate (see PDL Coating)
- Disposables: conical tubes and pipet tips

METHODS

1. Prepare E-18 primary cortical neuron cell suspension comprising 5.33 x 10^6 cells/ml in the seeding medium in a 50 ml conical tube. The seeding medium is Neurobasal + 2% B-27 + 1% G-5 + 0.5 mM GlutaMAX.

2. Transfer 1 ml of glial cells (2.67 x 10^6 cells/ml) and 1 ml of neural cells (5.33 x 10^6 cells/ml) to a 50 ml conical tube. The total live cell density at seeding is 4 x 10^6 cells/ml at 2:1 cell ratio of neurons to glia.

3. Seed cells in a desired volume into the SEEDEZ™ by dispensing near the center of the substrates.

4. Cover the plate and transfer to a 5% CO_2 37ºC incubator for 30 minutes; this allows cells to start adhering.

5. Transfer the plate to hood and add 250 µl of medium per well.

6. Cover the plate and transfer to a 5% CO_2 37ºC incubator for culturing.

7. Feed every 2-3 days by exchanging approximately half the medium.

1-WEEK CULTURING RESULTS

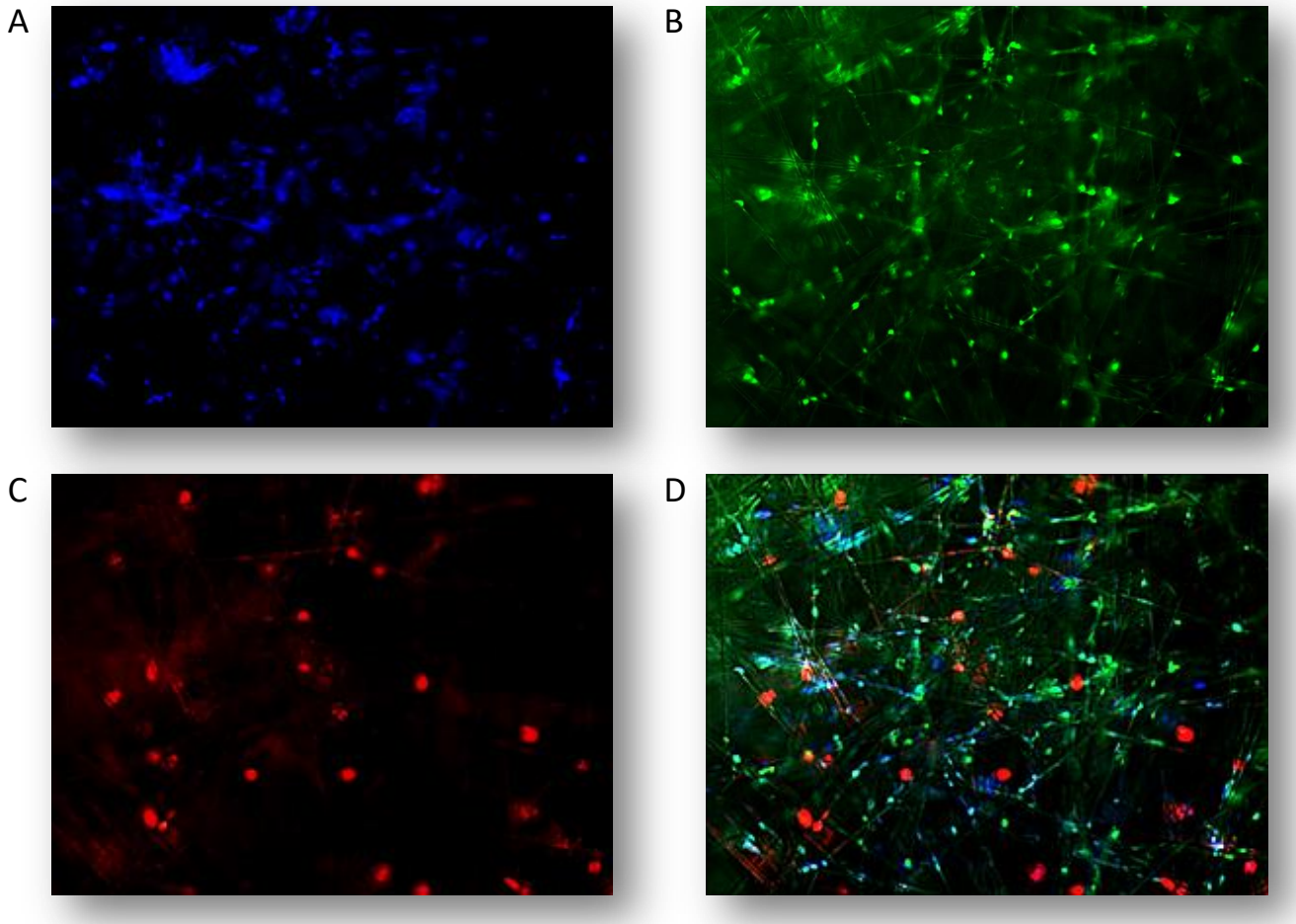


Fig. 6 Mixed culture of 2:1 neurons: mixed glia (at plating) in PDL-coated SeedEZ SC-C048 substrates at 1 week.

E-18 harvested neurons and P0-harvested and 1X passaged mixed glia (astrocytes and microglia; ~15% microglia in a total glia population) were seeded at 4 x 10^6 cells/ml in 30 µl into PDL-coated (0.1 mg/ml) SeedEZ SC-C048 substrate.

Cells were cultured for one week. During first 3 days in culture the medium composition was Neurobasal + 2% B-27(-AO) + 1% G-5 + 0.5 mM L-glutamine. At day 4 in culture the medium composition was changed to Neurobasal + 2% B-27(-AO) + 0.5 mM GlutaMAX. Cultures were fed every 2-3 days by exchanging approximately half the medium.

On Day 8, cell were stained using live cell stains and imaged on Nikon Eclipse 80i at 10X:

A. Calcein Blue AM labeled cells. By itself, Calcein Blue AM stains cytoplasm of all cells blue.
B. 5-(and-6)-Carboxyfluorescein Diacetate labeled cells. The stain is predominantly selective for neurons, glia expel it.
C. Alexa Fluor® 568 isolectin GS-IB4 conjugate. The stain is selective for microglia.
D. Overlapped images showing Calcein Blue AM, 5-(6) CFDA and IB4 stained cells all in one image.

RESULTS SHOW THE FOLLOWING:

I. *Difficult to culture primary cell types* can be successfully cultured in a 3D cell culture in the SEEDEZ™.

II. *Mixed cell populations* can be seeded and successfully cultured in 3D in the SEEDEZ™.

III. Common Poly-D-Lysine coating "sticks" to the SEEDEZ™ and provides for good cell adhesion.

IV. Adherent, difficult to culture cell types can be cultured successfully in the SEEDEZ™, if the SEEDEZ™ is coated with appropriate cell adhesive ligands.

V. SEEDEZ™ is transparent.

VI. *Cells can be imaged in the SEEDEZ™* using a standard fluorescence microscope.

VII. The culture was indeed three-dimensional. First, the top of the culture was imaged. Next, the SEEDEZ™ comprising 3D cell culture was flipped and the bottom side of the culture was imaged. Both the top and the bottom side of the culture were imaged and had live cells.

VIII. Live cells can be selectively labeled in the SEEDEZ™ and imaged in the SEEDEZ™.

IX. In general, live cell stains can be used at different time points during culturing in the SEEDEZ™ to assess 3D culture health and function.

X. Following 1 week in culture, the SEEDEZ™ remains stable and rigid enough that a 3D cell culture embedded in the SEEDEZ™ can be handled using sterile tweezers. For example, you may easily flip the substrate to image from both sides to resolve cell distribution through the culture thickness or transfer the culture to another dish or any other cell ware disposable.

PROTOCOL: The seeding of 3D mixed cultures in an extracellular matrix into uncoated SEEDEZ™

The protocol describes how to seed mixed 3D cell cultures of brain cells in a sol-state extracellular matrix suspension into uncoated SEEDEZ™ SC-C048 substrates in a 48-well plate. * The mixed population of cells comprised primary cortical neurons, and secondary (1X passaged) astrocytes and microglia. At seeding, the cell ratio was 2:1 neuron:glia at a total live cell density of 4 x 10^6 cells/ml in 7.5 mg/ml final extracellular matrix protein concentration.

** Courtesy of Dr. Laplaca's Lab, Coulter Department of Biomedical Engineering, Georgia Institute of Technology*

THE PROTOCOL DOES NOT PROVIDE DETAILS OF E-18 PRIMARY CORTICAL NEURON ISOLATION AND DISSOCIATION, AND P0-P1 ASTROCYTE/MIXED GLIA ISOLATION. THESE PROTOCOLS ARE AVAILABLE AT:

http://www.ncbi.nlm.nih.gov/pubmed/17409489

Cullen DK, Vukasinovic J, Glezer A, Laplaca MC.

Microfluidic engineered high cell density three-dimensional neural cultures.

J Neural Eng. 2007, 4(2):159-72.

ALTERNATIVE CELL SOURCES:

Cells, cortices, and whole brains may be obtained from vendors such as BrainBits. E-18 cortices may also be used as glial cell source. For ordered cells/ tissues, please follow isolation and maintenance protocols provided by the cell/ tissue supplier.

MATRIGEL ALIQUOTING AND STORAGE OF SINGLE USE VIALS

MATERIALS

- Growth Factor Reduced Matrigel, High Concentration; BD Biosciences Product No. 354263
- Hank's Balanced Salt Solution (HBSS)
- Micropipette; preferably, a positive displacement micropipette for Matrigel dispensing
- Disposables: pipette tips, capillary pistons if a positive displacement micropipette is used, and conical tubes

METHODS

Store Matrigel at -20°C prior to aliquoting. DO NOT STORE IN FROST-FREE FREEZER. ALL ALIQUOTING SHOULD BE DONE AT ONCE. Aliquot into one time use aliquots. FOR ALL STEPS KEEP MATRIGEL ON ICE. USE PRE-CHILLED REAGENTS, PIPET TIPS AND OTHER DISPOSABLES. Matrigel gels above 10°C. HANDLE USING STERILE TECHNIQUES:

1. Prior to aliquoting, thaw Matrigel on ice in the back of a 4°C refrigerator overnight.
2. Pre-chill sterile HBSS, sterile pipet tips, and sterile 50 ml conical tubes.
3. Transfer thawed vial of Matrigel into the cell culture hood (the solution will be viscous).
4. Dilute to 15 mg/ml using ice-cold HBSS.

5. Swirl to mix.
6. Aliquot 1.5 ml into ice-cold 50 ml conical tubes using pre-chilled pipette tips.
7. Store at -20°C.

MAINTENANCE AND DISSOCIATION OF P0-HARVESTED MIXED GLIAL CELLS PRIOR TO SEEDING

MATERIALS

- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12)
- Fetal Bovine Serum (FBS)
- Phosphate-Buffered Saline Ca^{++} and Mg^{++} free (PBS) 1X
- 0.25%Trypsin-EDTA
- Seeding medium: Neurobasal Medium, B-27 Supplement, G-5 Supplement, GlutaMAX
- HBSS, Trypan Blue and hemocytometer for cell counting
- Disposables: T-75 flasks, conical tubes, serological pipettes, and micropipette tips

METHODS

1. Dissociate two neonatal cortices and plate into a T-75 flask in 10 ml of medium DMEM/F-12 + 10% FBS.
2. Feed cells every 2-3 days thereafter, and passage or dissociate for seeding as follows:
3. Transfer 0.25% Trypsin-EDTA (1X) and medium to a 37°C water bath to warm up.

   If dissociating for seeding in Matrigel, do not warm up the medium.
4. Aspirate medium from the flask.
5. Add 10 ml of 1X PBS to the flask and gently rinse the cells. Aspirate PBS.
6. Add 3 ml of 0.25% Trypsin-EDTA (1X) and gently tilt the flask to ensure that the entire bottom surface is covered.
7. Transfer flask to incubator for 3-5 minutes.
8. Gently tap the side of the flask to detach cells. Observe cells under the microscope.

   If most cells are detached, transfer the flask to hood.

   If cells are still adhered, place the flask back to incubator for 1 minute; repeat this step until cells are detached.
9. Add 7 ml of medium (DMEM/F-12 + 10% FBS) to the flask to neutralize the Trypsin.
10. Use 10 ml serological pipette and rinse the bottom of the flask with medium/Trypsin mixture repeatedly to collect as many cells as possible.

11. Transfer cell suspension to a 50 ml conical tube and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).

12. Remove supernatant and re-suspend cell pellet.

13. Count cells using hemocytometer and Trypan Blue exclusion.

14. Add seeding medium to have total glia live cell density of 5.33 x 10^6 cells/ml in this step.

    The seeding medium is Neurobasal + 2% B-27 + 1% G-5 + 0.5 mM GlutaMAX.

    Glial cell solution is added to neural cell solution prior to seeding, and the combined cell solution added to an equal volume of 15 mg/ml Matrigel prior to seeding into the SEEDEZ™.

SEEDING

MATERIALS

- Cells: neurons, astrocytes and microglia
- Matrigel GFR at a protein concentration of 15 mg/ml (see Matrigel Aliquoting)
- Medium: Neurobasal, B-27 Supplement, G-5 Supplement, GlutaMAX
- Disposables: SEEDEZ™ SC-C048, 48-well plate, conical tubes, pipette tips, and capillary pistons if positive displacement micropipette is used

METHODS

One day before neuron/glia seeding:

1. Transfer a tube of 15 mg/ml Matrigel from the -20°C freezer into a 4°C refrigerator; keep Matrigel on ice.

2. Thaw for at least 24 hours.

On the day of cell seeding:

KEEP CELLS SOLUTIONS, PIPET TIPS, AND ALL DISPOSABLES IN CONTACT WITH MATRIGEL ON ICE.

3. Place SC-C048 SEEDEZ™ substrates into a 48-well plate.

4. Prepare E-18 primary cortical neuron cell suspension of 10.66 x 10^6 cells/ml in the seeding medium (Neurobasal + 2% B-27 + 1% G-5 + 0.5 mM GlutaMAX) in a 50 ml conical tube.

5. Take an ice-cold 50 ml conical tube and add 0.5 ml of glial cells in the seeding medium to 0.5 ml of neural cells in the seeding medium. The live cell density in this solution is 8 x 10^6 cells/ml. Keep cell solution on ice.

6. Take an ice-cold 50 ml conical tube and add 1 ml of 15 mg/ml to an equal volume of cell suspension from Step 5.
   The final total live cell density in this solution is 4 x 10^6 cells/ml at a final Matrigel concentration of 7.5 mg/ml.

7. Swirl tube to mix or pipette up and down to homogenize the cell solution; do not trap bubbles.

8. Keep the 50 ml conical tube comprising cells in 7.5 mg/ml Matrigel on ice.
9. Use ice cold pipette tips to dispense cells in a sol-state 7.5 mg/ml Matrigel into the SEEDEZ™.
10. Seed cells in a desired volume into the SEEDEZ™ by dispensing near the center of the substrates.

    You may dispense using regular or positive displacement micropipette. Pipette cell solution up and down before dispensing into the SEEDEZ™; do not trap bubbles.
11. Cover the plate and transfer into a 5% CO_2 37°C incubator for 45 minutes for Matrigel to gel.
12. Transfer the plate into the hood and add 250 µl of seeding medium per well.
13. Return plate to incubator for culturing.
14. Feed every 2-3 days by exchanging approximately half the medium.

2-WEEK CULTURING RESULTS

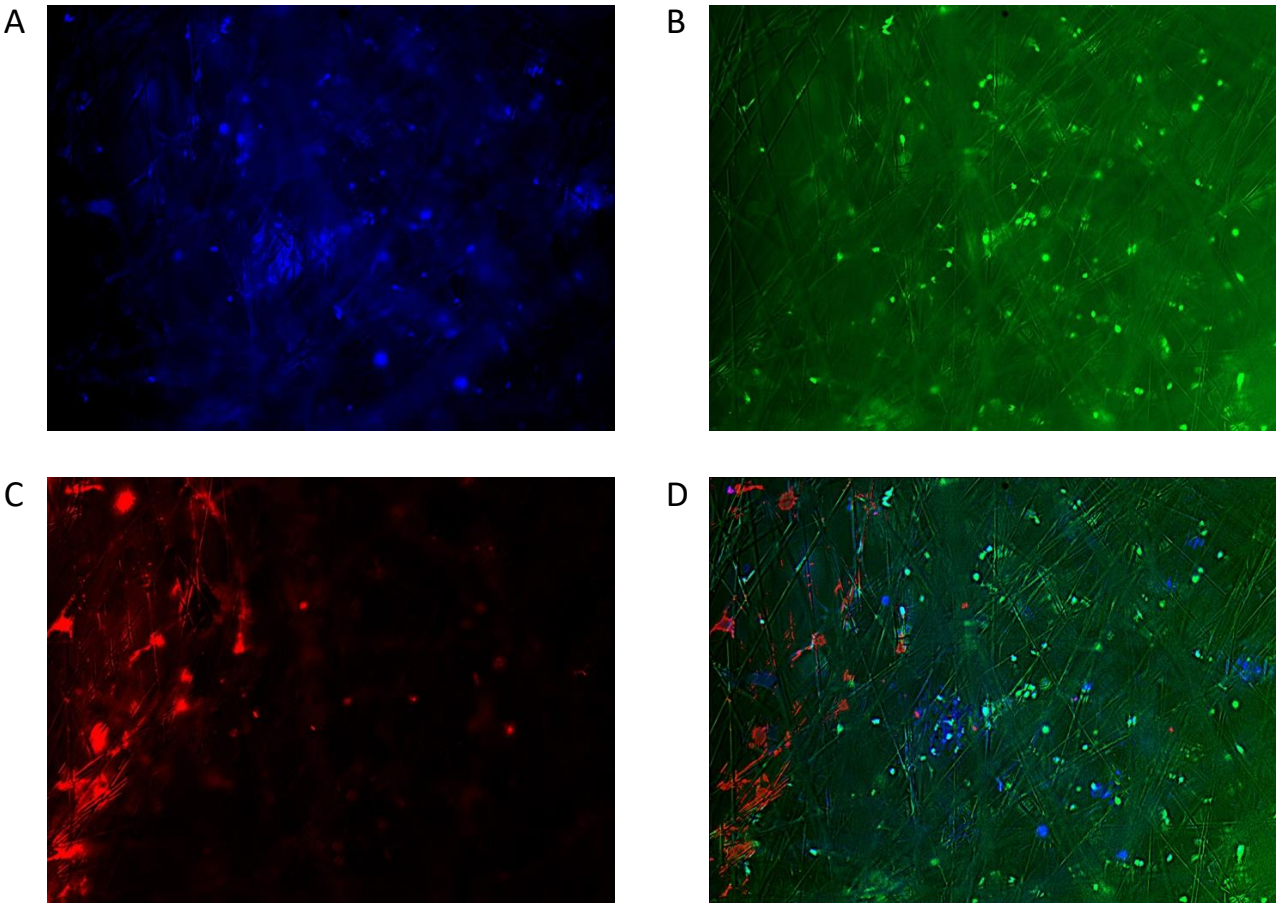


Fig. 7 Mixed culture of 2:1 neurons: mixed glia (at plating) in GFR Matrigel in uncoated SeedEZ SC-C048 at 2 weeks.

E-18 harvested neurons and P0-harvested and 1X passaged mixed glia (astrocytes and microglia; ~15% microglia in a total glia population) were seeded at 4 x 10^6 cells/ml in 7.5 mg/ml growth factor reduced Matrigel at a total volume of 30 µl into uncoated SeedEZ SC-C048 substrate.

Cells were cultured for two weeks. During first 3 days in culture the medium composition was Neurobasal + 2% B-27(-AO) + 1% G-5 + 0.5 mM GlutaMAX. At day 4 in culture the medium was changed to Neurobasal + 2% B-27(-AO) + 0.5 mM GlutaMAX. Cultures were fed every 2-3 days by exchanging approximately half the medium.

On Day 15, cell were stained using live cell stains and imaged on Nikon Eclipse 80i at 10X:

A. Calcein Blue AM labeled cells. By itself, Calcein Blue AM stains cytoplasm of all cells blue.
B. 5-(and-6)-Carboxyfluorescein Diacetate labeled cells. The stain is predominantly selective for neurons .
C. Alexa Fluor® 568 isolectin GS-IB4 conjugate. The stain is selective for microglia.
D. Overlapped images showing Calcein Blue AM, 5-(6) CFDA and IB4 stained cells all in one image.

RESULTS SHOW THE FOLLOWING:

I. *SEEDEZ™ wicks proteinaceous extracellular matrices.*

II. Adherent cells can be cultured successfully in *uncoated* SEEDEZ™, if the cells are seeded in an appropriate sol-state extracellular matrix into the SEEDEZ™.

III. *Difficult to culture primary cell types* can be successfully cultured in a 3D gel cell culture in the SEEDEZ™.

IV. *Mixed cell populations* can be seeded and successfully cultured in a 3D gel cell culture in the SEEDEZ™.

V. *Cells can be imaged in the gel in the SEEDEZ™* using a standard fluorescence microscope.

VI. SEEDEZ™ is transparent.

VII. *Cells can be imaged in the SEEDEZ™* using a standard fluorescence microscope.

VIII. The culture was indeed three-dimensional. First, the top of the culture was imaged. Next, the SEEDEZ™ comprising 3D cell culture was flipped and the bottom side of the culture was imaged. Both the top and the bottom side of the culture were imaged and had live cells.

IX. Live cells can be selectively labeled in the SEEDEZ™ and imaged in the SEEDEZ™.

XI. In general, live cell stains can be used at different time points during culturing in the SEEDEZ™ to assess 3D culture health and function.

XII. Following 2 weeks in culture, the SEEDEZ™ remains stable and rigid enough that a 3D cell culture embedded in the SEEDEZ™ can be handled using sterile tweezers. For example, you may easily flip the substrate to image from both sides to resolve cell distribution through the culture thickness or transfer the culture to another dish or any other cell ware disposable.

PROTOCOL: The seeding of adherent cells in a diluted ECM into uncoated and coated SEEDEZ™

The protocol describes how to seed primary-harvested and one-time passaged glial cells in a low protein concentration sol-state extracellular matrix suspension into uncoated- and Poly-D-Lysine coated SEEDEZ™ SC-C048 substrates in a 48-well plate. Glial cells were 1X passaged astrocytes and microglia. At seeding, the total live cell density was 2.5 x 10^6 cells/ml in 3.75 mg/ml protein concentration Matrigel extracellular matrix.

THE PROTOCOL DOES NOT PROVIDE DETAILS OF P0-P1 ASTROCYTE/MIXED GLIA ISOLATION. THESE PROTOCOLS ARE AVAILABLE AT: http://www.ncbi.nlm.nih.gov/pubmed/17409489

Cullen DK, Vukasinovic J, Glezer A, Laplaca MC.

Microfluidic engineered high cell density three-dimensional neural cultures.

J Neural Eng. 2007, 4(2):159-72.

ALTERNATIVE CELL SOURCES:

Cells, cortices, and whole brains may be obtained from vendors such as BrainBits. E-18 cortices may also be used as glial cell source. For ordered cells/ tissues, please follow isolation and maintenance protocols provided by the cell/ tissue supplier.

MATRIGEL ALIQUOTING AND STORAGE OF SINGLE USE VIALS

MATERIALS

- Growth Factor Reduced Matrigel, High Concentration; BD Biosciences Product No. 354263
- Hank's Balanced Salt Solution (HBSS)
- Micropipette; preferably, a positive displacement micropipette for Matrigel dispensing
- Disposables: pipette tips, capillary pistons if a positive displacement micropipette is used, and conical tubes

METHODS

Store Matrigel at -20°C prior to aliquoting. DO NOT STORE IN FROST-FREE FREEZER. ALL ALIQUOTING SHOULD BE DONE AT ONCE. Aliquot into one time use aliquots. FOR ALL STEPS KEEP MATRIGEL ON ICE. USE PRE-CHILLED REAGENTS, PIPET TIPS AND OTHER DISPOSABLES. Matrigel gels above 10°C. HANDLE USING STERILE TECHNIQUES:

1. Prior to aliquoting, thaw Matrigel on ice in the back of a 4°C refrigerator overnight.
2. Pre-chill sterile HBSS, sterile pipette tips, and sterile 50 ml conical tubes.
3. Transfer thawed vial of Matrigel into cell culture hood (the solution will be viscous).
4. Swirl to mix.
5. Aliquot 1.5 ml into ice-cold 50 ml conical tubes using pre-chilled pipette tips.
6. Store at -20°C.

POLY-D-LYSINE COATING

MATERIALS

- Poly-D-lysine Hydrobromide MW >300 kDa lyophilized powder, γ-irradiated; Sigma-Aldrich Product No. P7405
- Sterile, de-ionized water
- Disposables: SEEDEZ™ substrates SC-C048, 48-well plates, micropipette tips, conical tubes

METHODS

1. Prepare 100 µg/ml Poly-D-Lysine coating solution in sterile DI water. Store at 2-8 °C.
2. Place SC-C048 SEEDEZ™ substrates into a 48-well plate inside the hood.
3. Add 250 µl of 100 µg/ml Poly-D-Lysine solution to each well comprising SEEDEZ™.
4. Cover the plate by lid and transfer to incubator for at least 6 hours. Overnight incubation recommended.
5. Transfer the plate to hood and aspirate PDL.
6. Rinse the substrates 2X with sterile DI water.
7. After you aspirate the last rinse, dry the SEEDEZ™ substrate by placing the pipette tip on the substrate and aspirating any remaining liquid from the SEEDEZ™. You will notice the color change from transparent to white when dried.
8. Cover the plate. PDL-coated SEEDEZ™ substrates are ready for use.

MAINTENANCE AND DISSOCIATION OF P0-HARVESTED GLIAL CELLS PRIOR TO SEEDING

MATERIALS

- Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12)
- Fetal Bovine Serum (FBS)
- Phosphate-Buffered Saline Ca^{++} and Mg^{++} free (PBS) 1X
- 0.25%Trypsin-EDTA
- Disposables: T-75 flasks, conical tubes, serological pipettes, and micropipette tips
- HBSS, Trypan Blue and hemocytometer for cell counting

METHODS

1. Dissociate two neonatal cortices and plate into a T-75 flask in 10 ml of DMEM/F-12 + 10% FBS medium.
2. After 3 days, check for confluency. Next, aspirate the medium and add 10 ml of fresh medium.
3. Feed cells every 2-3 days thereafter, and passage or dissociate for seeding as follows:
4. Transfer 0.25% Trypsin-EDTA (1X) and medium to a 37ºC water bath to warm up.

If dissociating for seeding in Matrigel, do not warm up the medium.

5. Aspirate medium from the flask.
6. THIS STEP IS FOR DISSOCIATION PRIOR TO SEEDING ONLY:

   Add 10 ml of 1X PBS to the flask and gently rinse the cells. Aspirate PBS.
7. Add 3 ml of 0.25% Trypsin-EDTA (1X) and gently tilt the flask to ensure that the entire bottom surface is covered.
8. Transfer the flask to incubator for 3-5 minutes.
9. Gently tap the side of the flask to detach cells. Observe cells under the microscope.

   If most cells are detached, transfer the flask to hood.

   If cells are still adhered, place the flask back to incubator for 1 minute; repeat this step until cells are detached.
10. Add 7 ml of medium (DMEM/F-12 + 10% FBS) to the flask to neutralize Trypsin.
11. Use 10 ml serological pipette and rinse the bottom of the flask with medium/Trypsin mixture repeatedly to collect as many cells as possible.
12. Transfer cell suspension to a 50 ml conical tube and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).
13. Remove supernatant and re-suspend cell pellet in:

    FOR SEEDING INTO THE SEEDEZ™: 1 ml of medium (DMEM/F-12 + 10% FBS).

    FOR PASSAGING: 2 ml of DMEM/F-12 + 10% FBS.
14. Count cells using hemocytometer and Trypan Blue exclusion.
15. FOR SEEDING: Add seeding medium to have total glia live cell density of 5 x 10^6 cells/ml in this step.
    Glial cell solution is added to an equal volume of 7.5 mg/ml Matrigel at seeding.

    FOR PASSAGING: Split 2 ml of cell suspension into three T-75 flasks and add DMEM/F-12 + 10% FBS to have 10 ml per flask.

SEEDING

MATERIALS

- Cells: glia (astrocytes and microglia, or astrocytes depending on maintenance)
- Reagents: Matrigel GFR at a protein concentration of 15 mg/ml (see Matrigel Aliquoting), HBSS
- Medium: DMEM/F-12 + 10% FBS
- Disposables: SEEDEZ™ SC-C048, 48-well plate, PDL-coated SEEDEZ™ in a 48-well plate (see PDL Coating), conical tubes, and micropipette tips

METHODS

One day before cell seeding:

1. Transfer a tube of 15 mg/ml Matrigel from the -20°C freezer into a 4°C refrigerator; keep Matrigel on ice.
2. Thaw for at least 24 hours.

On the day of cell seeding:

KEEP CELLS SOLUTIONS, PIPET TIPS, AND ALL DISPOSABLES IN CONTACT WITH MATRIGEL ON ICE.

3. Place SC-C048 SEEDEZ™ substrates into a 48-well plate (Plate A). Transfer to freezer to pre-chill, if necessary.

   Use Plate A for seeding cells in a low concentration Matrigel into *uncoated* SEEDEZ™ substrates.

4. Transfer PDL-coated SC-C048 SEEDEZ™ substrates in a 48-well plate (Plate B) to freezer to pre-chill, if necessary.

   Use Plate B for seeding cells in a low concentration Matrigel into *PDL-coated* SEEDEZ™ substrates.

5. Dilute 15 mg/ml Matrigel to 7.5 mg/ml using HBSS.
6. Swirl to mix and keep on ice.
7. Take a 50 ml conical tube and add 1 ml of glial cells in the medium to 1 ml of 7.5 mg/ml Matrigel.

   The live cell density in this solution is 2.5 x 10^6 cells/ml at a final Matrigel concentration of 3.75 mg/ml.

8. Swirl tube to mix and keep on ice.
9. Use ice cold pipet tips to dispense cells in a sol-state 3.75 mg/ml Matrigel into the SEEDEZ™ substrates in Plate A, and into the PLD-coated SEEDEZ™ substrates in plate B.
10. Seed cells in a desired volume into the SEEDEZ™ by dispensing near the center of the substrates.

    Pipette up and down to homogenize cell solution before dispensing into the SEEDEZ™; do not trap bubbles.

11. Cover the plates and transfer into 5% CO_2 37ºC incubator for 45 minutes for Matrigel to gel.
12. Transfer the plates to hood and add 250 µl of medium per well.
13. Return plate to incubator for culturing.
14. Feed every 2-3 days by exchanging approximately half the medium.

1-DAY CULTURING RESULTS

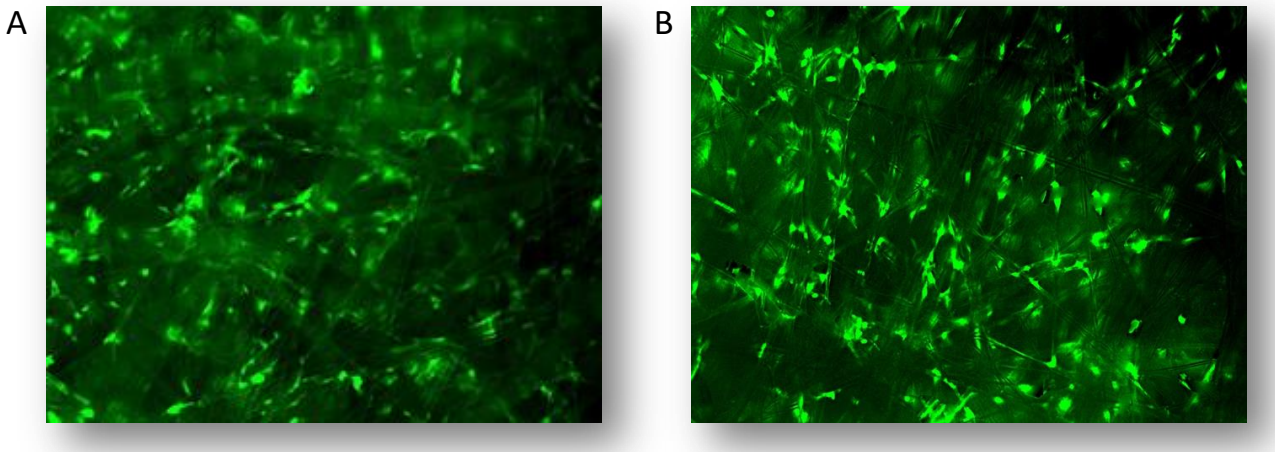


Fig. 8 1-day cultured mixed glia; 2.5 x 10^6 cells/ml in 3.75 mg/ml GFR Martigel seeded into the SeedEZ SC-C048. 1X passaged P0-harvested glia were cultured for 1day in DMEM/F-12 + 10% FBS. Cells were stained by Calcein AM (live cells) and imaged using Nikon Eclipse 80i at 10X.

A. 45 µl cell suspension seeded in an uncoated SeedEZ substrate SC-C048.
B. 30 µl cell suspension seeded in a Poly-D-Lysine- coated (0.1 mg/ml) SeedEZ substrate SC-C048.

RESULTS SHOW THE FOLLOWING:

I. *Primary-harvested and one-time passaged cells* can be cultured short-term in 3D in a low protein concentration gel in the SEEDEZ™. *** Without the SEEDEZ™, low protein concentration Matrigel may not form stable gel for long-term cell support in a 3D cell culture*.

II. Sol-state extracellular matrix cell suspension permeates through uncoated SEEDEZ™ substrate (see Fig. 8A) and Poly-D-Lysine coated SEEDEZ™ substrate (see Fig. 8B).

In a separate study, the cells were seeded in 8 mg/ml sol-state Matrigel into uncoated and PDL-coated SEEDEZ™ substrates. The cell suspension permeated both the uncoated and PDL-coated SEEDEZ™ substrates.

III. Adherent cells can be cultured without pre-coating SEEDEZ™ with cell adhesive molecules, if cells are seeded into the SEEDEZ™ in an appropriate sol-state extracellular matrix even when the matrix does not form a stable gel for long-term cell culture (see Fig. 8A).

In a separate study, with these and other cell types, cells were seeded in 1 mg/ml protein Matrigel suspension into uncoated SEEDEZ™. At 1 mg/ml protein, Matrigel did not gel. Still, cells were cultured successfully long-term (up to 4 weeks at the time of imaging).

IV. Adherent cells seeded in an extracellular matrix can be cultured in PDL-coated SEEDEZ™ (Fig. 8B).

V. SEEDEZ™ is transparent.

VI. *Cells can be imaged in the gel in the SEEDEZ™, and in the gel in the PDL-coated SEEDEZ™* using a standard fluorescence microscope.

VII. The cultures were indeed three-dimensional. First, the top of the culture was imaged. Next, the SEEDEZ™ comprising 3D cell culture was flipped and the bottom side of the culture was imaged. Both the top and the bottom side of the culture were imaged and had live cells.

*** At 3.75 mg/ml protein concentration, Matrigel may not form stable gel for long term 3D cell culture without the SEEDEZ™ substrate.* To maintain a gelled consistency, Matrigel manufacturer does not

recommend diluting the matrix to less than 3 mg/ml, and not less than 4 mg/ml to prevent incomplete gel formation. When less than 4 mg/ml Matrigel cell suspension is used at plating, beyond 7 days in culture and frequently earlier, the gel adhesion to cellware disposables is reduced, the gelled consistency is reduced and further degraded and disrupted in routine media exchanges.

WHEN SEEDING CELLS IN OR ON TOP OF MATRIGEL USE SEEDEZ™. The SEEDEZ™ continues to provide for adherent cell support in 3D cultures under these conditions (see 10-Day Culturing Results in a serum-free cell environment, next).

10-DAY CULTURING RESULTS IN A SERUM-FREE MEDIUM FOLLOWED BY STARVING

In this Example, cells were seeded in a low protein concentration Growth Factor Reduced Matrigel ECM in uncoated SEEDEZ™ substrates and cultured in a serum-free medium (DMEM/F-12) for 1 week without any media exchanges. After 1 week, the medium was aspirated. The culture was overlaid by another SEEDEZ™ substrate saturated with fresh medium (DMEM/F-12) in 3.75 mg/ml Matrigel. Under these conditions, cells were cultured for another 3 days in a 5% CO_2 37 ^oC humidified incubator without any media additions other than that provided by an overlaid, relatively nutrient-rich, SEEDEZ™ substrate.

As can be seen in Fig. 9, at Day 11 in culture, the gel was substantially degraded. Still, the cells grew, extended processes, and formed 3D cell networks in the SEEDEZ™. Primary-harvested, secondary cells survived even though they were starved, the extracellular matrix gel degraded, and the SEEDEZ™ was not coated with cell adhesive ligands prior to cell seeding in a sol-state Matrigel.

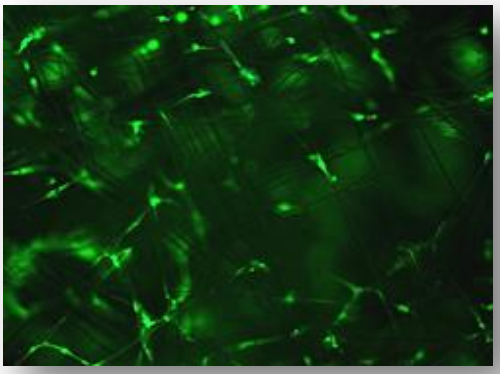


Fig. 9 3-Day starved (10 days in culture) mixed glia in a SeedEZ stack.

1X passaged mixed glia at 2.5 x 10^6 cells/ml in 3.75 mg/ml GFR Martigel were seed into an uncoated SeedEZ SC-C048 substrate in a 30 µl spot.

Cells were cultured **serum-free** in DMEM/F-12 for 1 week without media exchange. Next, the medium was aspirated and another wet SC-C048 SeedEZ substrate comprising DMEM/F-12 in 3.75 mg/ml GFR Matrigel was placed on top of the culture; no additional media was added. The culture was transferred to a humidified incubator for another 3 days.

At day 11, cells were stained using Calcein AM (live cells labeled green) and imaged on Nikon Eclipse 80i at 10X.

RESULTS SHOW THE FOLLOWING:

I. *Primary-harvested and one-time passaged brain cells* can be successfully cultured for 10 days even when seeded in an uncoated SEEDEZ™ substrate, in a degrading ECM gel, without serum, and with low nutrient availability.

   Even under these conditions the cell morphology in the SEEDEZ™ was round, cells extended long processes, and used the SEEDEZ™ substrate to form 3D cell networks.

II. Cells can be deprived of medium for a period of time not to exceed few days, if embedded in a hydrogel in the SEEDEZ™ when the hydrogel comprises "trapped" medium.

III. After 10 days in 3D cell culture, 3.75 mg/ml Matrigel deteriorated.

   FOR CULTURING IN A LOW PROTEIN CONCENTRATION MATRIGEL (LESS THAN 4-5 mg/ml), USE THE SEEDEZ™ TO SUPPORT CELLS IN LONG TERM CULTURE.

IV. Cells can be cultured in a SEEDEZ™ substrate and covered by another SEEDEZ™ substrate.

V. Cells can be deprived of medium for a few days even when embedded in an unstable gel in the SEEDEZ™, provided that the culture is overlapped by another SEEDEZ™ substrate which previously absorbed medium.