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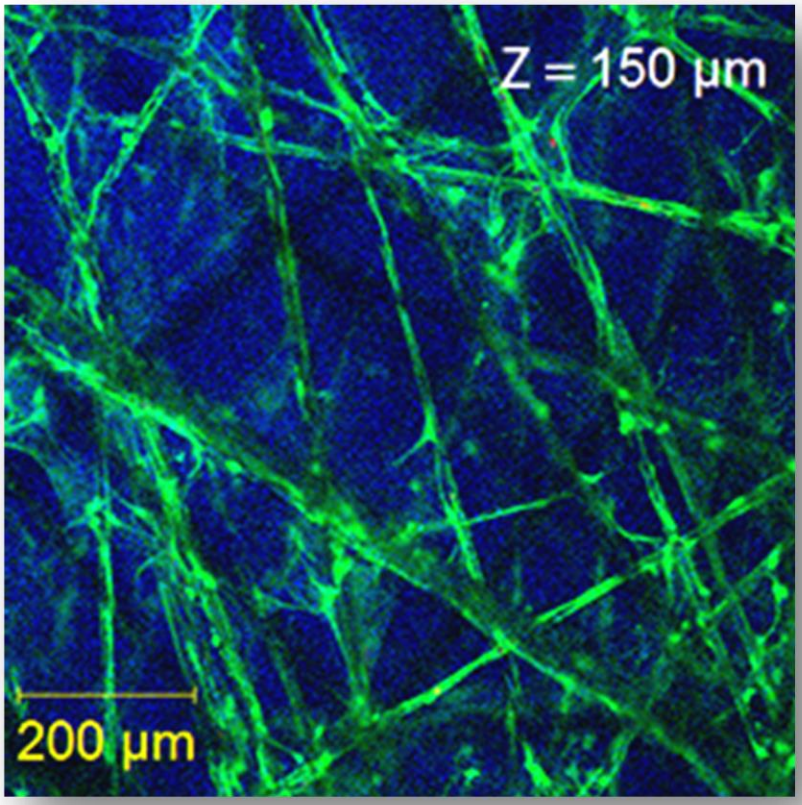
SEEDEZ™ PROTOCOLS

METHODS AND PROTOCOLS FOR CELL RECOVERY FROM 3D CELL CULTURES EMBEDDED IN THE



SEEDEZ™

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Recovery of Cells Embedded in the SeedEZ™

December 2018

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INTRODUCTION

Cells recovered from three-dimensional (3D) cell cultures cultured in the SeedEZ™ may be used to assess yield, viability, function and phenotype. Cells may be matured, pre-conditioned, drug- or otherwise treated in the SeedEZ™. After recovery, cells may be sub-cultured in the SeedEZ™ or in 2D, used in mixed cultures, biochemical assays, molecular assays, or counted.

GENERAL GUIDELINES FOR RECOVERY OF ADHERENT CELLS EMBEDDED INTO COATED SEEDEZ™

Adherent cells seeded into the SeedEZ™ coated with cell adhesive ligands may be recovered after days and weeks in a 3D cell culture using proteolytic enzymes and chelating agents and their combinations.

TO RECOVER CELLS FROM THE SEEDEZ™ CONTINUE TO USE PROTOCOLS NORMALLY USED FOR CELL DETACHMENT FROM FLASKS, AND OTHER CELL-WARE AND CULTURE-WARE DISPOSABLES.

The following guidelines may assist in optimizing or troubleshooting your current protocols:

A. Cell adhesion molecules which mediate cell binding with the other cells and the extracellular matrix depend on Calcium and other divalent cations.

    a. Do not use buffered saline *with Calcium and Magnesium* for washing cells when cell dissociation or cell recovery is the objective. Calcium and Magnesium generally promote cell attachment and adhesion.

    b. Washing cells in Phosphate-Buffered Saline (PBS) or Dulbecco's Phosphate-Buffered Saline (DPBS) *without Calcium and Magnesium* reduces cell adhesion.

    c. EDTA (ethylenediaminetetraacetic acid) chelates divalent cations and reduces cell adhesion.

    d. EGTA (ethylene glycol tetraacetic acid) chelates divalent cations but with a higher affinity for Calcium than for Magnesium ions.

    e. To improve cell recovery rates of strongly adherent cells you may need a combination of reagents, for example, PBS-EDTA in place of PBS for washing, and Trypsin-EDTA in place of Trypsin for dissociation.

B. The choice of chelating agents and proteolytic enzymes for cell dissociation and recovery may be influenced by cell adhesive ligands used to coat the SeedEZ™ substrates.

C. A combination of two reagents in a one-step or a two-step process may yield more complete cell recovery than a single reagent at a higher concentration.

    a. For strongly adherent cells seeded into the SeedEZ™, you may use a reagent which cleaves the coating and a reagent which cleaves other cell adhesion proteins. For example, use Collagenase for Collagen coating and Trypsin to cleave cell adhesion proteins at arginine and lysine residues.

    b. If Trypsin-EDTA is used, Trypsin attacks proteins at arginine and lysine residues and EDTA chelates divalent cations disrupting those cell adhesion molecules which depend on those cations to function. For example, EDTA may disrupt cadherins which need Calcium and cell-cell adhesion is reduced. EDTA also influences integrins which need Calcium, Magnesium and other divalent cations in mediating cell-to-matrix adhesion.

c. If cells were cultured in a 3D cell culture for weeks and secreted their endogenous matrix support which composition is very different from the coating applied to SeedEZ™, you may need two reagents; a reagent which cleaves the coating, and a reagent which cleaves the matrix.

D. Mechanical agitation may improve the success of cell recovery. Mechanical agitation may include manual tube inversion, vortexing or the use of orbital shakers, rockers and rotators.

E. If Trypsin is used:

a. For dissociation of cells cultured in serum containing medium, wash cells twice using PBS before adding Trypsin; serum inactivates Trypsin.

b. For cell recovery, do not use serum-free medium to inactivate Trypsin. Trypsin may be inactivated using serum containing medium or Trypsin neutralizing solution. However, if serum concentration is too low, Trypsin may not be inactivated completely.

REAGENTS FOR THE RECOVERY OF ADHERENT CELLS EMBEDDED IN THE COATED SEEDEZ™

For recovery of adherent cells cultured in the SeedEZ™ coated by cell adhesive molecules, the choice of proteolytic enzymes and/or chelating agents for cell detachment, dissociation and isolation depends on:

A. Coating composition

B. Methods by which the coating is applied

C. Coating degradation (hydrolytic, enzymatic digestion and cell activity)

D. Cell types

E. Cell seeding density

F. Medium composition

G. Culturing period

The above factors influence all or some of the following: cell-to-cell adhesion, cell adhesion to the coating, cell adhesion to secreted endogenous matrix constituents, adhesion of the coating to the SeedEZ™, and temporal degradation of the coating, if any.

The cell recovery reagents are typically selected based on the cell type and molecules used to coat the SeedEZ™ for adhering cells. However, cell recovery reagents commonly used for a monolayer culture on a coated plastic dish are also used for the recovery of cells from the coated SeedEZ™ substrates.

Commonly used cell recovery reagents are:

- Versene (1 X PBS/ 0.5mM EDTA) for gentle non-enzymatic cell dissociation and recovery of less adherent cell types
- Trypsin, a serine protease
- Trypsin-EDTA
- Alternatives to Trypsin such as Papain or TrypLE

- Collagenase
- Accutase
- Dispase
- Combinations of the above

GENERAL GUIDELINES FOR RECOVERY OF CELLS EMBEDDED IN A HYDROGEL IN THE SEEDEZ™

Cells seeded into the SeedEZ™ in an extracellular matrix gel or any other hydrogel may be recovered using chemicals and/or physical methods contributing to gel de-polimerization; proteolytic enzymes, chelating agents and their combinations.

TO RECOVER CELLS FROM A GEL EMBEDDED INTO UNCOATED SEEDEZ™ FOLLOW THE PROTOCOLS USED FOR CELL ISOLATION FROM THE GEL IN QUESTION.

TO RECOVER CELLS FROM A GEL EMBEDDED INTO COATED SEEDEZ™ FOLLOW THE PROTOCOLS USED FOR CELL ISOLATION FROM THAT GEL WHEN ADHERED TO THE COATED LABWARE.

The following guidelines may assist in optimizing or troubleshooting your current protocols:

A. Cell adhesion molecules which mediate cell binding with the other cells and the extracellular matrix depend on Calcium and other divalent cations.

    a. Do not use buffered saline *with Calcium and Magnesium* for washing cells when cell dissociation or cell recovery is the objective. Calcium and Magnesium generally promote cell attachment and adhesion.

    b. Washing cells in Phosphate-Buffered Saline (PBS) or Dulbecco's Phosphate-Buffered Saline (DPBS) *without Calcium and Magnesium* reduces cell adhesion.

    c. EDTA (ethylenediaminetetraacetic acid) chelates divalent cations and reduces cell adhesion.

    d. EGTA (ethylene glycol tetraacetic acid) chelates divalent cations but with a higher affinity for Calcium than for Magnesium ions.

    e. To improve cell recovery rates of strongly adherent cells you may need a combination of reagents, for example, PBS-EDTA in place of PBS for washing, and Trypsin-EDTA in place of Trypsin for dissociations.

B. The choice of chelating agents, proteolytic enzymes, and hydrogel de-polimerization methods for cell detachment, dissociation and recovery may be influenced by:

    a. Cell adhesive molecules used to coat the SeedEZ™ substrates prior to seeding cells in a sol-state gel.

    b. The type of hydrogel used to embed cells in the SeedEZ™ in a sol-state hydrogel suspension.

C. If cells were cultured for weeks, it is likely that the composition of extracellular matrix at the time of cell recovery will be quite different from the exogeneous matrix embedded in the SeedEZ™ at the time of seeding (and especially when the cell types in question are known to secrete their own endogenous matrix support).

Under said conditions, a regent typically used for cell recovery from the exogeneous matrix may be less efficient. You may need a different reagent; a reagent which recovers cells from the endogenous matrix, or you may need two reagents; a reagent which recovers cells from the exogenous matrix and a reagent which recovers cells from the endogenous matrix, applied together or sequentially for more complete cell recovery.

D. A combination of two reagents in a one-step or a two-step process may yield more complete cell recovery than a single reagent at a higher concentration.

    a. For strongly adherent cells seeded into the SeedEZ™, you may use a reagent which cleaves the coating and a reagent which cleaves other cell adhesion proteins. For example, Collagenase for Collagen coating and Trypsin, cleaving cell adhesion proteins at arginine and lysine residues.

    b. If Trypsin-EDTA is used, Trypsin attacks proteins at arginine and lysine residues and EDTA chelates divalent cations disrupting those cell adhesion molecules which depend on those cations to function. For example, EDTA may disrupt cadherins which need Calcium and cell-cell adhesion is reduced. EDTA also influences integrins which need Calcium, Magnesium and other divalent cations in mediating cell-to-matrix adhesion.

    a. If cells were cultured in a 3D cell culture for weeks and secreted their endogenous matrix support which composition is very different from the coating applied to SeedEZ™ and the gel embedded into the SeedEZ™ at cell seeding, you may need more than one reagent; a reagent which cleaves the coating, and reagents which cleave specific matrix constituents.

E. If Trypsin is used:

    a. For dissociation of cells cultured in serum containing medium, wash cells twice using PBS before adding Trypsin; serum inactivates Trypsin.

    b. For cell recovery, do not use serum-free medium to inactivate Trypsin. Trypsin may be inactivated using serum containing medium or Trypsin neutralizing solution. However, if serum concentration is too low, Trypsin may not be inactivated completely.

F. Mechanical agitation may improve success of cell recovery. Mechanical agitation may include manual inversion of tube containing cells in a gel in the SeedEZ™, vortexing, or use of orbital shakers, rockers and rotators.

G. Pressure or vacuum may also be used to assist in a more complete cell recovery, provided that flow indeed passes through the gel and not around it.

H. Cells recovered from a gel may be more "sticky".

    Cells recovered from a gel may be more "sticky" than cells recovered from planar substrates even when cell recovery solution de-polymerizes gel efficiently. FOR FLOW CYTOMETRY AND CELL SORTING APPLICATIONS, WASH CELLS COPIOUSLY, IF RECOVERED FROM A GEL.

REAGENTS FOR RECOVERY OF CELLS EMBEDDED IN A HYDROGEL IN THE SEEDEZ™

For recovery of cells cultured in an extracellular matrix or gel embedded the SeedEZ™, the choice of proteolytic enzymes, chelating agents, or physical methods for gel de-polymerization and cell isolation depends on:

A. The SeedEZ™ coating, if any

B. Gel composition and concentration at plating

C. Gel and/or coating degradation (hydrolytic, enzymatic digestion and cell activity)

D. Cell types

E. Cell seeding density

F. Culturing period

The above factors influence all or some of the following: cell-to-cell adhesion, cell adhesion to the coating, cell adhesion to the gel constituents, cell adhesion to secreted endogenous matrix constituents, adhesion of the coating to the SeedEZ™, adhesion of the gel to the coated or uncoated SeedEZ™, and temporal degradation of the gel, and the coating, if any.

The following guidelines may assist in selecting or adjusting cell recovery solution for your needs:

A. Understand the composition of hydrogel used; there are many proteolytic enzymes available.

B. Low concentration gel typically de-polymerizes easier than a high concentration gel.

C. If the concentration is too low, a sol-state gel solution may not gel, or may not form a stable gel after plating.

Cell recovery reagents commonly used for cell isolation from a hydrogel are also used for cell isolation from that hydrogel embedded in the SeedEZ™.

Commonly used cell recovery solutions from gels include:

- BD Cell Recovery Solution, or Dispase, for cell recovery from Matrigel
- Collagenase, Collagenase/Trypsin, or Collagenase/Trypsin-EDTA
- Hyaluronidase or Collagenase/Hyaluronidase
- Agarose
- EDTA or Citrate buffer for Alginate de-polymerization
- Trypsin, Trypsin-EDTA, or Accutase
- Combinations of the above

WHAT TO EXPECT FROM A GEL IN LONG-TERM CULTURE AND HOW THAT AFFECTS RECOVERY?

Consider the following when choosing gel de-polymerization and cell recovery method after long-term cell culture:

A. Gel contraction.

Gels may contract as a result of cell activity.

GEL CONTRACTION MAY COMPLICATE CELL RECOVERY FROM GEL-BASED 3D CELL CULTURES

B. Enzymatic and hydrolytic degradation.

Gels degrade by diverse mechanisms depending on their chemistry, concentration, cell activity and environment. Typically, they lose mass or dissolve by processes which reverse the gelling mechanism

such as enzymatic and hydrolytic degradation. Cell mediated matrix remodeling and digestion by invasive cells is also a factor.

GEL DEGRADATION MAY FACILITATE CELL RECOVERY FROM GEL-BASED 3D CELL CULTURES

C. Gel remodeling by cells.

Depending on cell types, cell seeding densities, maturity, and a culturing period, expect a degree of extracellular matrix or gel remodeling as cells secrete endogenous extracellular matrix constituents or degrade the exogenous matrix. *The hydrogel composition may be very different at the time of cell seeding and at the time of cell recovery.*

GEL REMODELLING MAY COMPLICATE CELL RECOVERY

CONSIDERING HYDROGEL CONTRACTION AND DEGRADATION, SEEDING CELLS IN A HYDROGEL IN THE SEEDEZ™ MAKES YOUR 3D CELL CULTURES MORE CONSISTENT WHILE STILL PROVIDING FOR EXCELLENT CELL RECOVERY RATES

FOR REPRODUCIBLE RESULTS USE SEEDEZ™

PROTOCOL: The recovery of cells embedded in Poly-D-Lysine coated SeedEZ™

SeedEZ™ is not cell adhesive. Without cell adhesive molecules, cells clump in the SeedEZ™. Poly-D-Lysine coating is commonly used to improve adhesion of neuronal cell lines, primary neuron, and glial cells. Trypsin is efficient for cell recovery from Poly-D-Lysine-coated SeedEZ™. Trypsin cleaves peptides on the C-terminal side of lysine and arginine amino acid residues.

Poly-D-Lysine (PDL) is the substrate of Trypsin. Accordingly, 0.25% Trypsin- 1 mM EDTA treatment provides for high rates of cell recovery from PDL-coated SeedEZ™ substrates. Using the below Materials and Methods, after 1 day in culture, the cell recovery exceeded 70% of viable P0-harvested and 1X passaged mixed glial cells seeded into PDL-coated SeedEZ™ substrates.

For cell seeding into Poly-D-Lysine coated SeedEZ™, please see SeedEZ™ Cell Seeding Protocols.

MATERIALS

- Cells

  Glia (astrocytes and microglia) for the below Results using the below enzymatic and chelating reagents

- Poly-D-Lysine coated SC-C048 SeedEZ™ substrates comprising cells
- A 48-well plate with the above SeedEZ™ substrates seated one per well
- Medium

  For the below Results with glial cells: DMEM/F-12 + 10% FBS

  DMEM/F-12: Dulbecco's Modified Eagle Medium: Nutrient mixture F-12

  FBS: Fetal Bovine Serum

- Sterile Phosphate-Buffered Saline (PBS) 1X Calcium and Magnesium free
- 0.25%Trypsin-EDTA; Life Technologies Trypsin 0.25% in 1 mM EDTA Product No. 25200
- HBSS, Trypan Blue and a hemocytometer for cell counting

- Sterile tweezers, 6” or longer for ease of access to the bottom of the 50 ml conical tube to retrieve the substrate
- Sterile disposables: conical tubes, serological pipettes, and micropipette tips

METHODS

1. Aspirate and discard medium from SC-C048 SeedEZ™ substrates seated in a 48-well plate.
2. Rinse the substrates 2X with PBS; aspirate the last rinse.
3. Place SC-C048 SeedEZ™ substrate in a 50 ml conical tube using tweezers; a substrate per tube.

   Do not place more than one SC-C048 SeedEZ™ substrate in a 50 ml conical tube.

   Multiple substrates will be stacked on top of each other. This creates a longer path for cells to pass in order to exit the substrates, and the cell recovery rate may be significantly reduced.
4. Add 2 ml of 0.25% Trypsin-EDTA pre-warmed to 37ºC.
5. Place the conical tube in a 37ºC water bath for 5-10 minutes.

   Shorter trypsinization gives higher cell viability but potentially lower number of recovered cells.
6. Add 5 ml of serum containing medium to neutralize Trypsin.
7. Swirl to mix.
8. Spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).

   RCF is the relative centrifugal force. The actual RPM depends on the centrifuge model and rotor used:

   RCF = $1.11824396 \times 10^{-5} \times r \times N^2$ where r = rotational radius in cm, and N = rotational speed in RPM
9. Remove the SeedEZ™ from the conical tube using tweezers. Be careful not to disturb the pellet.

   The advantage of a 50 ml conical tube is that the cone keeps SC-C048 SeedEZ™ substrate horizontal and above the cell pellet after spin down while allowing easy access to the substrate for its removal using tweezers.

   Transfer the SeedEZ™ substrates to a new flat-bottom 48-well plate for subsequent staining and imaging to check for completeness of cell recovery, if so desired (see Step 16).
10. Aspirate supernatant and leave approximately 50 µl.
11. Re-suspend cell pellet in the approximate 50 µl volume if cell count (see Step 12) is the objective.

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.

    You may re-suspend in larger volume; however, for accurate hemocytometer count from each SeedEZ™ substrate do not dilute too much as you may have too few cells per chamber if the seeding density was low (< 10^6 cells/ml) and the cells were post-mitotic; or the cells did not proliferate owing to their phenotype, medium composition or a short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Re-suspend cell pellet in an appropriate volume of medium for re-plating or use cells for biochemical and molecular studies; for example, to extract RNA or nuclear proteins. Most of these studies; however, do not require cell recovery and can be conducted *in situ* in the SeedEZ™.
12. FOR CELL COUNT FROM EACH SEEDEZ™ SUBSTRATE:

Use a 10-100 µl variable pipette and estimate the total volume in the tube.

FOR AN AVERAGED CELL COUNT FROM PLURALITY OF SEEDEZ™ SUBSTRATES:

Combine cell solutions, and then estimate the total volume.

13. Take 10 µl of cell suspension, add HBSS and Trypan Blue and vortex; calculate your dilution factor.
14. Load 10 µl to each chamber of a hemocytometer.
15. Wait a few minutes for cells to settle and count.
16. To check for completeness of cell recovery, stain and image the cells (if any) in the SeedEZ™ substrates in a flat-bottom multi-well plate into which the substrates were transferred to following spin down.

VORTEXING SEEDEZ™ SUBSTATES DURING AND EVEN AFTER TRYPSINIZATION MAY RESULT IN SEEDEZ™ "DELAMINATION" INTO TWO OR MORE THINNER SUBSTRATES (THIS IS MORE PRONOUNCED FOR POLY-D-LYSINE COATED SUBSTRATES). SEEDEZ™ "DELAMINATION" FACILITATES CELL RECOVERY.

RESULTS

The recovery of P0 harvested and 1X passaged mixed glial cells (astrocytes and microglia) seeded at 2.5 x 10^6 cells/ml exceeded 70% of live cells (adhered to the SeedEZ™ at plating) after 1 day in culture. Over 90% of recovered cells were viable after 5-minute long trypsinization. The result is an averaged value obtained from 4 SeedEZ™ substrates, with respective 4 cell pellets, re-suspended, combined and mixed in a homogeneous solution. The cells were counted using hemocytometer and Trypan Blue exclusion method.

Prior to cell seeding, SeedEZ™ substrates were coated with 100 µg/ml Poly-D-Lysine solution in sterile DI water by overnight incubation in a 5% CO_2 37°C incubator. The molecular weight of Poly-D-Lysine was greater than 300 KDa. The cells were cultured in DMEM/F-12 + 10% FBS in PDL-coated SeedEZ™.

For cell seeding protocols in Poly-D-Lysine coated SeedEZ™ substrates, please see SeedEZ™ Cell Seeding Protocols.

RESULTS SHOW THE FOLLOWING:

I. Although the SeedEZ™ is a three-dimensional substrate and not a flat dish, it still allows successful recovery of cells embedded in it and cultured in 3D in approximately 400 µm thickness.

II. Using the protocol, Poly-D-Lysine coated SeedEZ™ allowed successful recovery of adherent cells.

III. Using the protocol, over 70% of live cells seeded in the PDL-coated SeedEZ™ were recovered after 1 day in culture.

IV. Using the protocol, over 90% of all recovered cells were viable.

YOUR CELL RECOVERY RATE MAY BE HIGHER OR LOWER DEPENDING ON:

A. Cell types.
B. Cell seeding densities.
C. The number of days in culture.
D. Medium composition including serum and growth factors, if any.

E. Molecular weight of Poly-D-Lysine.

F. Poly-D-Lysine (PDL) concentration in a solution.

G. The duration of SeedEZ™ incubation in a PDL solution.

HOW TO AUTOMATE CELL RECOVERY FROM THE SEEDEZ™ AND INCREASE THROUGHPUT?

The following guidelines are for automated, and semi-automated, parallel cell recovery from SeedEZ™ substrates, a substrate per well of the multi-well plate. The below materials, equipment and methods provide for cell pellets, one per well of the multi-well plate, for subsequent cell counting, biochemical assays, molecular studies or sub-culturing.

RECOMMENDATIONS

USE CONICAL-BOTTOM OR V-BOTTOM MULTI-WELL PLATES FOR CELL RECOVERY. These plates allow to easily separate SeedEZ™ substrates from respective cell pellets. After spin, SeedEZ™ substrates are typically seated horizontally and trapped in the "cone" at a vertical distance from the cell pellet which is seated at the bottom of the "cone." This allows easy substrate removal without disturbing the cell pellet at the bottom of the well.

IF CONICAL- OR V-BOTTOM PLATES ARE UNAVAILABLE IN A DESIRED FORMAT, USE REACTION (PCR) PLATES AND SEAL THEM FOR SPIN. The PCR plates are available in a 24-well, 48-well, 96-well format and higher. As PCR well-diameters may be smaller than those of cell culture plates of the same format, check well diameter before deciding which PCR plate to use. (Note that most PCR plates are made of polypropylene and can be sterilized by steam autoclaving. Ask the plate supplier for recommended parameters. Some suppliers recommend 121° C for 15 minutes at 15 psi.)

Flat-bottom multi-well plates are not recommended.

Flat-bottom plates may not yield a complete cell recovery following spin. The SeedEZ™ removal from respective wells after spin may also trap cells.

Multi-well inserts in a multi-well plate, or integral multi-well insert systems are not recommended.

Inserts typically comprise membranes which pores are too small and in insufficient number for complete cell isolation for most cell types when the SeedEZ™ substrates are seated in the insert.

MATERIALS

- Cells
- Poly-D-Lysine coated SC-C048 SeedEZ™ substrates comprising cells
- 48-well plate with the above SeedEZ™ substrates seated one per well
- Reagents: serum containing medium or medium and Trypsin neutralizing solution, Phosphate-Buffered Saline (PBS) 1X without Calcium and Magnesium, 0.25%Trypsin-EDTA (Life Technologies Product No. 25200)
- Disposables: *conical-bottom or V-bottom multi-well plates* in place of 50 ml conical tubes, strip caps or silicone rubber sealing mats if PCR plates are used, and micropipette tips (long tips)

EQUIPMENT

Cell recovery may be automated, and throughput increased for cell isolation from separate SeedEZ™ substrates seated in the wells of the multiwall plate using the following equipment:

- A centrifuge with a rotor capable of carrying and spinning multi-well plates.

  If unskirted PCR plates are used, semi-skirted of fully skirted PCR plates may be used as adapters, if necessary. Alternatively, use dedicated PCR plate adapters, or removable PCR tube tray holders with lids provided they fit into the swing-bucket rotor used for spinning down the plates.

- Sterilization equipment for non-sterile plates and lids, if sterile conditions are used in subsequent cell studies.

  If using steam autoclave for non-sterile polypropylene PCR plates, polypropylene strip caps or silicone caps and mats, first run a test cycle using sterilization parameters you commonly use. Polypropylene is generally considered autoclavable; however, inspect the plates and caps for warping at the end of the cycle. If silicone is used, inspect silicone caps or mats. Ensure that at the end of the cycle, the caps/mats still fit into the plates.

- Vacuum source and a vacuum aspirator for multi-well plates.

  After spin, use automated tools or multi-channel pipettes and adapters to place the SeedEZ™ substrates towards the side of the wells without vacuum. Next, apply suction to lift the substrates and transfer.

  You may also use hand-held vacuum aspirator with a multi-channel adapter; for example, an 8-channel adapter.

  In the event you cannot lift the substrates using suction, check your vacuum source. It may be too weak, or the system has leaks. If you cannot aspirate the medium, you will not be able to lift the substrates either.

METHODS

1. Aspirate and discard medium from SC-C048 SeedEZ™ substrates seated in a 48-well plate.
2. Rinse SeedEZ™ substrates seated in a multi-well plate twice with PBS; aspirate the last rinse.
3. Transfer SeedEZ™ substrates to a conical-bottom multi-well plate.

   For SeedEZ™ SC-C048 substrates, transfer the substrates to a 48-well or a *24-well PRC plate.

   * Some PCR plates may be thick-walled. Ensure that 9.5 mm in diameter SeedEZ™ SC-C048 substrate fits into the well of the 48-well plate. Alternatively, use a 24-well PCR plate.

4. Add 0.25% Trypsin-EDTA pre-warmed to 37°C.
5. Seal the plate and transfer to a 37°C 5% CO_2 incubator for 5-10 minutes.

   Shorter trypsinization gives higher cell viability but potentially lower number of recovered cells.

6. Transfer the plate to sterile hood and add at least 2X Trypsin volume in Step 4 in medium to neutralize Trypsin.

   Depending on plate used and volume used in Step 4, you may have to aspirate an amount of Trypsin and then add medium. If you aspirate an amount of Trypsin, do so from the free surface to avoid cell aspiration.

7. Seal the plate and transfer to centrifuge, balance, and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).

8. After spin down, transfer the plate to hood.

9. Push the SeedEZ™ substrates towards the side of the well (be careful not to disturb cell pellets at the bottom of the conical wells). Use pipet tips, but do not use suction.

10. Use suction to transfer the SeedEZ™ substrates to another plate (see Step 14).

11. Aspirate supernatant (leave approximately 50 µl).

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.

12. Re-suspend cell pellets in the approximate 50 µl volume, if cell count (see Step 13) is the objective.

    You may re-suspend in larger volume; however, for accurate cell count from each SeedEZ™ substrate do not dilute too much as you may have too few cells if the seeding density was low (< 10^6 cells/ml) and the cells were post- mitotic, or the cells did not proliferate owing to their phenotype, medium composition or a short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Re-suspend cell pellet in an appropriate volume of medium for re-plating or use cells for biochemical and molecular studies; for example, to extract RNA or nuclear proteins. Most of these studies; however, do not require cell recovery and can be conducted *in situ* in the SeedEZ™.

13. Count cells using methods you normally use.

14. To check for completeness of cell recovery, stain and image the cells (if any) in the SeedEZ™ substrates in a flat-bottom multi-well plate into which the substrates were transferred to following spin down.

PROTOCOL: The recovery of cells embedded in an extracellular matrix gel in the SeedEZ™

The protocol describes how to recover cells embedded in a Matrigel® extracellular matrix in the SeedEZ™.

Matrigel is the trade name for a gelatinous protein mixture secreted by Engelbreth-Holm-Swarm (EHS) mouse sarcoma and marketed by BD Biosciences and other companies under different trade names. Main components of Matrigel are structural proteins such as Laminin, Collagen IV, and Entactin which present cultured cells with adhesive peptide sequences they frequently encounter in their natural environment. Cell recovery from Matrigel may result in lower cell recovery rate than cell recovery from other hydrogels comprising one or fewer number of proteins (Collagen I, for example).

Several products are marketed for cell recovery from Matrigel. The below Materials and Methods use BD Biosciences Cell Recovery Solution. If other products are used, please follow manufacturer protocols. Depending on the seeding conditions, culturing conditions and a culturing period, the live cell recovery frequently exceeded 60% of brain cells seeded in Matrigel in the SeedEZ™ using the below Methods. The live cell recovery was greater than 35% of seeded cells even for difficult to culture brain cells after 2 weeks in culture, even when the cells were cultured without sera or growth factors other than those present in the Growth Factor Reduced (GFR) Matrigel (BD Product No. 354263), and even when the cells were seeded in a proteinaceous 7.5 mg/ml protein extracellular matrix in the SeedEZ™ with 83% of diverse Matrigel proteins gelled (or gelling) which complicated cell recovery.

For cell seeding in Growth Factor Reduced (GFR) Matrigel into the SeedEZ™, please see SeedEZ™ Cell Seeding Protocols.

MATERIALS

- Uncoated or Poly-D-Lysine coated SC-C048 SeedEZ™ substrates with cells embedded in GFR Matrigel matrix
- A 48-well plate with the above SeedEZ™ substrates seated one per well
- Cell culture medium
- Sterile Phosphate-Buffered Saline (PBS) 1X without Calcium and Magnesium
- Cell Recovery Solution; BD Biosciences Product No. 354253
- 0.25% Trypsin-EDTA; Life Technologies Product No. 25200, Trypsin 0.25% in 1 mM EDTA
- HBSS, Trypan Blue and a hemocytometer for cell counting
- Sterile tweezers, 6” or longer for ease of access to the bottom of the 50 ml conical tube to retrieve the substrate
- Sterile disposables: conical tubes, serological pipettes, and micropipette tips

METHODS

Part I: BD Cell Recovery Solution Treatment

FOR THE CELL RECOVERY SOLUTION TREATMENT PRE-CHILL CONICAL TUBES AND USE ICE-COLD PBS, CELL RECOVERY SOLUTION, AND MEDIUM. KEEP THE CONICAL TUBES ON ICE DURING ALL STEPS.

1. Aspirate and discard medium from SC-C048 SeedEZ™ substrates seated in a 48-well plate.

2. Rinse the substrates 2X with cold PBS; aspirate the last rinse.

3. Place SC-C048 SeedEZ™ substrate in a pre-chilled 50 ml conical tube using tweezers; a substrate per tube.

   Do not place more than one SC-C048 SeedEZ™ substrate in a 50 ml conical tube.

   Multiple substrates will be stacked on top of each other. This creates a longer path for cells to pass in order to exit the substrates, and the cell recovery rate may be significantly reduced.

4. Add at least 500 µl of cold BD Cell Recovery Solution per tube.

5. Vortex for 10 seconds.

6. Keep the tube on ice, and transfer to refrigerator for 2 hours.

7. Every half hour, take the tube and vortex for 10 seconds.

8. After 2 hours, transfer the tube to a 4ºC centrifuge and spin for 5 minutes at 1000 RCF (1000 RCF = 1000 x g).

9. Use long tweezers and transfer the SeedEZ™ substrate to a new 50 ml pre-chilled conical tube; keep the tube on ice.

   Do not disturb cell pellet below the SeedEZ™ substrate during SeedEZ™ removal.

10. Keep the tube on ice, aspirate supernatant and leave approximately 50 µl.

11. Re-suspend cell pellet in the approximate 50 µl volume if cell count (see Step 12) is the objective.

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.

    You may re-suspend in larger volume; however, for accurate hemocytometer count from each SeedEZ™ substrate do not dilute too much as you may have too few cells if your seeding density was low (< 10^6 cells/ml) and cells were post-mitotic, or cells did not proliferate owing to their phenotype, medium composition or short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Wash the pellet by gentle resuspension in ice cold PBS, followed by spin in a 4ºC centrifuge at 1000 RCF for 5 minutes. Aspirate supernatant and resuspend cells in an appropriate volume of medium. Combine the cells with cells from the Trypsin treatment (see Part II) and use for re-plating (if sterile conditions were maintained), or biochemical/ molecular studies; for example, to extract RNA or nuclear proteins.

12. FOR CELL COUNT FROM EACH SEEDEZ™ SUBSTRATE:

    Use a 10-100 µl variable pipette and estimate the total volume in the tube.

    FOR AN AVERAGED CELL COUNT FROM PLURALITY OF SEEDEZ™ SUBSTRATES:

    Combine cell solutions and then estimate the total volume.

13. Take 10 µl of cell suspension, add HBSS and Trypan Blue and vortex; calculate your dilution factor.

14. Load 10 µl to each chamber of a hemocytometer.

15. Wait a few minutes for cells to settle and count.

    The cell count shows effectiveness of cell isolation from Matrigel embedded in the SeedEZ™ using BD Cell Recovery Solution using this protocol.

Part II: 0.25% Trypsin – 1 mM EDTA Treatment

16. Add 2 ml of 0.25% Trypsin-EDTA pre-warmed to 37°C to a 50 ml conical tube to which the SeedEZ™ was transferred.

17. Vortex for 10 seconds.

18. Place the conical tube in a 37°C water bath for 5-10 minutes.

    Shorter trypsinization gives higher cell viability but potentially lower number of recovered cells.

19. Add 5 ml of medium to neutralize Trypsin.

20. Spin at a relative centrifugal force of 1000 RCF for 5 minutes at room temperature (1000 RCF = 1000 x g).

21. Take long tweezers and transfer the SeedEZ™ substrate to a new flat-bottom 48-well plate for subsequent staining and imaging, to check for completeness of cell recovery, if so desired (see Step 28).

    Do not disturb cell pellet below the SeedEZ™ substrate, during SeedEZ™ removal.

22. Aspirate supernatant and leave approximately 50 µl.

23. Re-suspend cell pellet in the approximate 50 µl volume, if cell count (see Step 24) is the objective.

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.

    You may re-suspend in larger volume; however, for accurate hemocytometer count from each SeedEZ™ substrate do not dilute too much as you may have too few cells if your seeding density was low (< 10^6 cells/ml) and cells were post-mitotic, or cells did not proliferate owing to their phenotype, medium composition or short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Wash the pellet by gentle resuspension in ice cold PBS, followed by 5-minute spin at 1000 RCF. Aspirate supernatant and resuspend cells in an appropriate volume of medium. Combine the cells with cells recovered from the Cell Recovery Solution treatment (see Part I) and use for re-plating (if sterile conditions were maintained), or biochemical/ molecular studies; for example, to extract RNA or nuclear proteins.

24. FOR CELL COUNT FROM EACH SEEDEZ™ SUBSTRATE:

    Use a 10-100 µl variable pipette and estimate the total volume in the tube.

    FOR AN AVERAGED CELL COUNT FROM PLURALITY OF SEEDEZ™ SUBSTRATES:

    Combine cell solutions, and then estimate the total volume.

25. Take 10 µl of cell suspension, add HBSS and Trypan Blue and vortex; calculate your dilution factor.

26. Load 10 µl to each chamber of a hemocytometer.

27. Wait a few minutes for cells to settle and count.

    If cells remained embedded in Matrigel in the SeedEZ™ following the Cell Recovery Solution treatment (see Part I), the cell count here shows the effectiveness of cell isolation from Matrigel embedded in the SeedEZ™ using a 2-hour BD Cell Recovery Solution treatment at 4°C, followed by 5-10 minute 0.25% Trypsin-EDTA treatment at 37°C.

28. To check for completeness of cell recovery, stain and image cells (if any) in the SeedEZ™ substrates in a flat-bottom multi-well plate into which the substrates were transferred to following spin down (see Step 21).

CELL RECOVERY RATE MIGHT BE FURTHER INCREASED USING 4°C TRYPSINIZATION AND SPIN. AT 4°C MATRIGEL REMAINS AT ITS SOL-STATE. THIS FACILITATES CELL RECOVERY IF THE GEL DID NOT FULLY DE-POLIMERIZE USING THE BD BIOSCIENCES CELL RECOVERY SOLUTION TREATMENT. FURTHER, AT 4°C TRYPSIN MAY BE APPLIED LONGER THAN 5-10 MINUTES WITH THE ADDED ADVANTAGE OF BEING GENTLER TO THE CELLS THAN AT 37°C.

RESULTS

CELL RECOVERY FROM A DILUTED ECM GEL IN THE SEEDEZ™ AFTER 1 DAY IN CULTURE

The live cell recovery of P0-harvested and 1X passaged glial cells seeded at 2.5 x 10^6 cells/ml in *3.75 mg/ml Matrigel* into *uncoated SeedEZ™* substrates exceeded 60% of live cells at seeding. At seeding, the dispensed volume of cells (astrocytes and microglia) in ice-cold Matrigel sol-state suspension was 30 µl per SeedEZ™ substrate SC-C048. *The cells were cultured for 1 day*. The medium composition was DMEM/F-12 + 10% FBS.

Cells were seeded in six SeedEZ™ replicates. After 1 day in culture, 3 replicates underwent 1-hour long Cell Recovery Solution treatment at 4°C and 3X vortexing lasting 10 seconds each. These samples were stained with Calcein AM and imaged (see Fig. 1A). The remaining 3 samples were treated as per the Part I and Part II

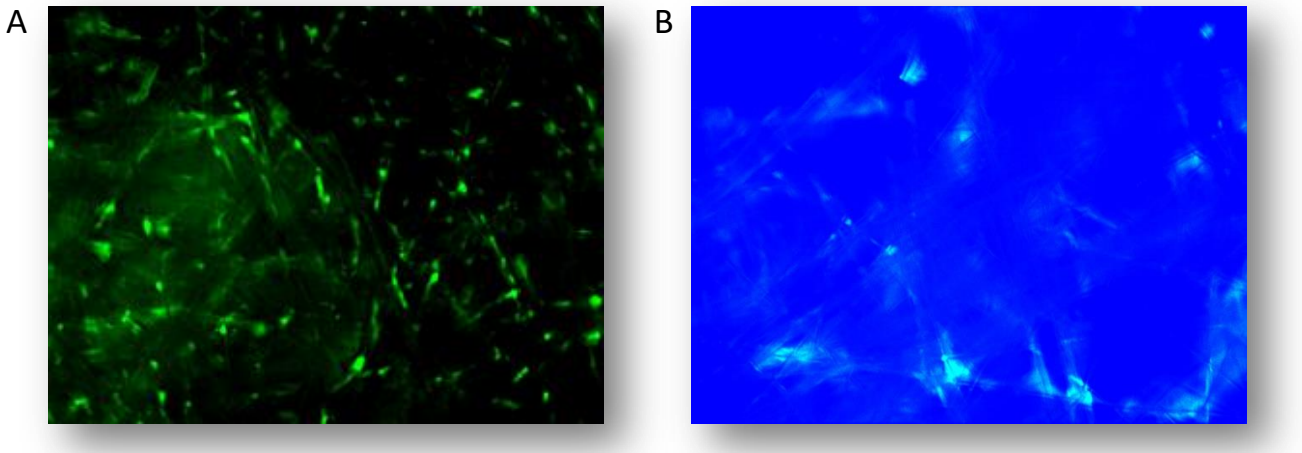

Fig. 1 1-day cultured mixed glial cells (astrocytes and microglia) in the SeedEZ following cell recovery treatments.

1X passaged P0-harvested cells were seeded at 2.5 x 10^6 cells/ml in 3.75 mg/ml GFR Martigel into the SeedEZ SC-C048 substrates in 30 µl volume. Cells were cultured for 1 day in DMEM/F-12 + 10% FBS. Following cell recovery treatments, the cells were stained and imaged in the SeedEZ using Nikon Eclipse 80i at 10X.

A. Calcein AM stained live cells following 1-hour Cell Recovery solution treatment. Cells were still present.
B. Calcein AM stained live cells and Hoechst 33342 stained nuclei of all cells after Cell Recovery Solution and Tryspin treatments. Light source intensity was increased to find cells. Very few live cells were seen with no dead cells.

of the above protocol (see Fig. 1B).

The samples which underwent 1-hour long Cell Recovery Solution treatment had live cells as shown in Fig. 1A. The cells were embedded in sections of not yet de-polymerized gel in the SeedEZ™, as was seen in phase contrast.

This outcome was consistent with a typical outcome of cell recovery for cells plated in GFR Matrigel without the SeedEZ™ after 1 hour of Cell Recovery Solution treatment in a 48-well plate, embedded in ice and placed on orbital shaker set at 150 RPM. Under these conditions, *7.5 mg/ml Matrigel without the SeedEZ™* typically remained adhered to the well as seen by lifting the plate up and looking at the plate bottom. Under these conditions, *3.75 mg/ml Matrigel without the SeedEZ™*

adhered to some wells but not all; still, it was still not fully de-polymerized even at a low 3.75 mg/ml Matrigel protein concentration for 3D cell culture applications without the SeedEZ™.

Note that as per BD Biosciences Cell Recovery Solution protocol (SPC-354253 Rev. 2.0 Release Date 9/21/01), a 1 mm thick layer of gelled Matrigel de-polymerized after 60 minutes on ice using BD Cell Recovery Solution. The Matrigel lot used here was different, the final GFR Matrigel protein concentration was typically between 3.75 mg/ml and 7.5 mg/ml, the gel comprised brain cells, and most importantly the Matrigel was used after a period in cell culture of 1 to 14 days in our studies. All these factors likely contributed to longer than anticipated Cell Recovery Solution treatment to recover cells than is the period suggested by the BD Biosciences Cell Recovery Solution protocol.

To increase cell yield, Lena Biosciences recommends at least 2-hour long BD Cell Recovery Solution treatment. You may find that that a shorter or longer period is needed to depolymerize Matrigel for your application. This will depend on Matrigel lot, protein concentration at seeding, cell types and cell seeding densities, medium and a culturing period for both cells seeded in Matrigel or cells seeded in Matrigel in the SeedEZ™.

As per the above protocol (Part I and Part II) Cell Recovery solution provided for 70% to 80% of all recovered cells after 2-hour long BD Cell Recovery Solution treatment, followed by 1000 RCF spin at 4°C. In a 2-hour treatment, the Cell Recovery Solution was efficient at recovering cells from 3.75 mg/ml Matrigel in the SeedEZ™ after 1 day in culture. The result is an averaged value obtained from 3 SeedEZ™ substrates, with respective 3 cell pellets, re-suspended, combined and mixed in a homogeneous solution. The cells were counted using hemocytometer and Trypan Blue exclusion method after the Cell Recovery Solution treatment, and the Trypsin treatment. Over 90% of all recovered cells were viable.

Using the protocol, the total live cell recovery yield exceeded 60% of cells seeded into the SeedEZ™ after 1 day in culture.

Following the above protocol (Part I and Part II) the SeedEZ™ substrates were stained by Calcein AM and Hoechst 33342 and imaged. As can be seen in Fig. 1B very few cells were present.

RESULTS SHOW THE FOLLOWING:

I. Although SeedEZ™ is a 3D substrate and not a flat dish, it allows successful seeding and recovery of cells embedded in it and cultured in 3D *in a gelled extracellular matrix*.

II. SeedEZ™ allows for cell recovery from a 3D gelled extracellular matrix embedded in it, *even when the gelled ECM comprises 3 extracellular matrix proteins*. THIS IS SIGNIFICANT BECAUSE NO SINGLE PROTEASE CLEAVES ALL MATRIGEL ECM PROTEINS IN A SHORT PERIOD OF TIME WHICH MAKES CELL RECOVERY COMPLEX.

III. Using the protocol, over 90% of all recovered cells were viable.

IV. Using the protocol, over 60% of live cells seeded in Matrigel in the SeedEZ™ were recovered after 1 day in culture.

YOUR CELL RECOVERY RATE MAY BE HIGHER OR LOWER DEPENDING ON:

A. Cell types.

B. Cell seeding densities.

C. Culturing period.

D. Medium composition including any sera and growth factors.

E. Matrigel concentration and Matrigel lot.

CELL RECOVERY FROM A DILUTED ECM GEL IN A PDL-COATED SEEDEZ™ AFTER 2 DAYS IN CULTURE

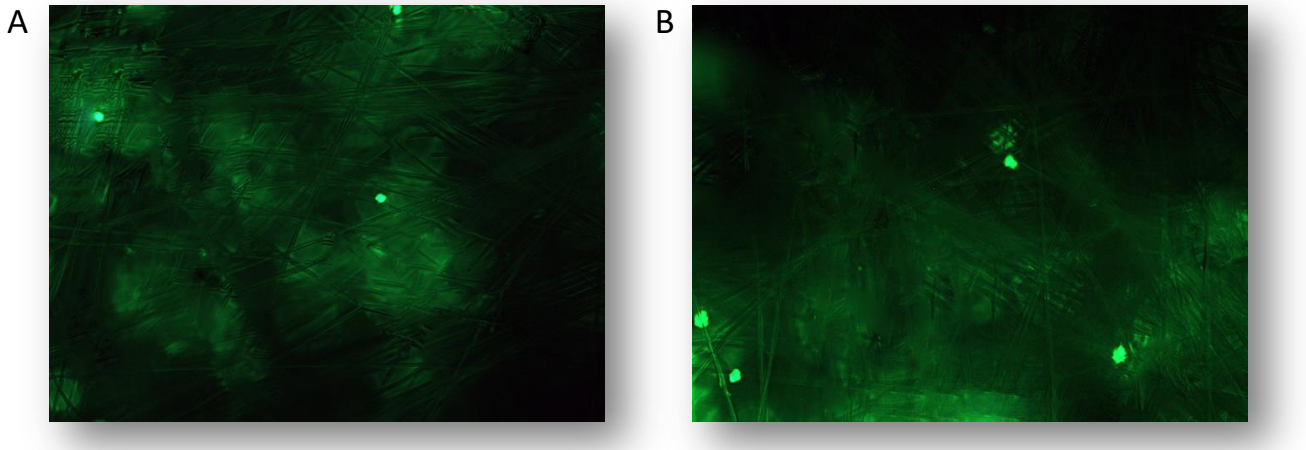

Fig. 2 2-day cultured mixed glial cells (astrocytes and microglia) in the PDL-coated SeedEZ following cell recovery.

1X passaged P0-harvested cells were seeded at 2.5 x 10^6 cells/ml in 3.75 mg/ml GFR Martigel into the Poly-D-Lysine coated SeedEZ SC-C048 substrates in 30 µl volume. Cells were cultured for 2 days in DMEM/F-12 + 10% FBS. Following cell recovery treatments, the cells were stained and imaged in the SeedEZ using Nikon Eclipse 80i at 10X.

A. Calcein AM stained live cells after cell recovery treatments in the SeedEZ. Very few live cells were present.
B. Same conditions as in **A** except that substrate **B** "delaminated" less than the substrate **A**.

Using the protocol, the recovery of P0-harvested and 1X passaged glial cells, seeded at 2.5 x 10^6 cells/ml in 3.75 mg/ml growth factor reduced Matrigel at a total volume of 30 µl in Poly-D-Lysine-coated SeedEZ™ resulted in nearly complete cell recovery. Cells were cultured for 2 days. The Cell Recovery solution provided for approximately 60% of all recovered cells. However, during 5-minute long trypsinization followed by vortexing, 4 out of 6 SeedEZ™ substrates "delaminated" into two or more thinner substrates; thus, allowing for nearly complete cell recovery after spin.

The result is an averaged value obtained from 6 SeedEZ™ substrates, with respective 6 cell pellets, re-suspended, combined and mixed in a homogeneous solution. The cells were counted using hemocytometer and Trypan Blue after Cell Recovery Solution treatment and Trypsin treatment. Using the protocol, the total live cell recovery exceeded 60% of cells seeded into the SeedEZ™ after 2 days in culture. Over 75% of all recovered cells were viable.

After cell recovery treatments, all SeedEZ™ substrates, including delaminated substrates, were stained using Calcein AM, but very few live cells were seen as shown in Fig. 2. The SeedEZ™ "delamination" facilitated cell recovery.

For cell seeding protocols in Poly-D-Lysine coated SeedEZ™ substrates, please see SeedEZ™ Cell Seeding Protocols.

RESULTS SHOW THE FOLLOWING:

I. Although the SeedEZ™ is a 3D cell culture substrate and not a flat dish, and although it is approximately 400 µm thick, it still allows *successful seeding and recovery of cells embedded and cultured in a* diluted but still *gelled extracellular matrix* (ECM) *in 3D in the SeedEZ™, even when the SeedEZ™ is coated.*

II. *SeedEZ™ allows for recovery of adherent cells from* a *gelled, 3D extracellular matrix* embedded in it, *even when the gelled ECM comprises 3 extracellular matrix proteins, and even when the SeedEZ™ is coated.*

III. Using the protocol, over 75% of all recovered cells were viable.

IV. Using the protocol, over 60% of live cells seeded in Matrigel in the PDL-coated SeedEZ™ were recovered after 2 day in culture.

YOUR CELL RECOVERY RATE MAY BE HIGHER OR LOWER DEPENDING ON:

A. Cell types.

B. Cell seeding densities.

C. The number of days in culture.

D. Medium composition including serum and growth factors, if any.

E. Matrigel concentration and Matrigel lot.

F. Molecular weight of Poly-D-Lysine.

G. Poly-D-Lysine (PDL) concentration in a solution.

H. The period of SeedEZ™ incubation in a PDL solution.

CELL RECOVERY FROM AN ECM GEL IN THE SEEDEZ™ AFTER 14 DAYS IN CULTURE

At seeding, 3D cultures comprised a 2:1 cell ratio of E-18 primary cortical neurons and P0-harvested and passaged glial cells (astrocytes and microglia). Glial cells were gently passaged once. There was 10%-40% microglia in a total glial population at seeding. The cells were seeded at $4x10^6$ cells/ml in 7.5 mg/ml GFR Matrigel in 30 µl volume into uncoated SeedEZ™ SC-C048 substrates. Cells were cultured for 14 days. During first 3 days in culture the medium composition was Neurobasal + 2% B-27 + 1% G-5 + 0.5 mM GlutaMAX. At day 4 in culture the medium composition was changed to Neurobasal + 2% B-27 + 0.5 mM GlutaMAX. Cultures were fed every 2-3 days by exchanging approximately half the medium.

Using the protocol, live cell recovery rate exceeded 35% of seeded cells.

After 14 days culture, the total cell number may have been higher or lower relative to what was seeded. Neurons were regarded as post-mitotic. Glial cells were not supplemented with G-5 after the first 3 days in culture. After 14 days in a 3D cell culture, cells were considered well anchored inside the extracellular matrix gel and the SeedEZ™ substrate. Still, even under these conditions, difficult to culture primary and secondary brain cells were isolated from 7.5 mg/ml GFR Matrigel extracellular matrix in the SeedEZ™, and the live cell number exceeded 35% of cells seeded into the SeedEZ™.

Using the protocol, the Cell Recovery solution provided for 2.5% cell recovery (average value from 6 cultures) relative to the total cell number at seeding. Out of 2.5% recovered cells, 1.4% were viable cells and 0.9% were dead cells. Subsequent, 10 minute long trypsinization provided for 55% cell recovery relative to what was seeded. Out of 55% recovered cells, 35% were viable cells and 20% were dead cells (percentages are with respect to the number of cells at seeding).

Using the protocol, the Cell Recovery Solution was not efficient in recovering cells from 7.5 mg/ml Matrigel embedded in the SeedEZ™ (< 2% live cells recovered with respect to what was seeded). The combination of Cell Recovery Solution and 0.25% Trypsin – 1 mM EDTA was more efficient (> 35% live cells recovered with respect to what was seeded).

RESULTS SHOW THE FOLLOWING:

I. Although the SeedEZ™ is a 3D substrate and not a flat dish, and although it is approximately 400 µm thick, it still allows successful recovery of adherent cells embedded in it and cultured in 3D in a gelled

extracellular matrix (ECM) at a protein concentration typical of a gel-based 3D culture without the SeedEZ™.

II. *SeedEZ™ allows for cell recovery from a gelled, 3D extracellular matrix* embedded in it, *even when the gel is 7.5 mg/ml protein rich and comprises a mixture of diverse ECM proteins including Laminin, Collagen IV, and Entactin.*

III. *Even primary-harvested and one-time passaged brain cells can be* successfully cultured and *recovered, even after 14 days in culture in a protein rich mixture of diverse 3D gelled ECM proteins in the SeedEZ™.*

IV. Using the protocol, after 14 days in culture, > 35% of live cells were isolated relative to what was seeded. The cells were primary/ secondary cells and the cells were isolated from a proteinaceous gel embedded in the SeedEZ™.

YOUR CELL RECOVERY RATE MAY BE HIGHER OR LOWER DEPENDING ON:

A. Cell types.
B. Cell seeding densities.
C. The number of days in culture.
D. Medium composition including serum and growth factors, if any.
E. Matrigel protein concentration at seeding.
F. Matrigel lot.

HOW TO AUTOMATE RECOVERY FROM MATRIGEL IN THE SEEDEZ™ AND INCREASE THROUGHPUT?

The following guidelines are for automated, and semi-automated, parallel recovery of cells embedded in gelled Matrigel extracellular matrix in the SeedEZ™ substrates, a substrate per well of the multi-well plate. The below materials, equipment and methods provide for cell pellets, one per well of the multi-well plate, for subsequent cell counting, biochemical assays, molecular studies or sub-culturing.

For cell seeding in Growth Factor Reduced (GFR) Matrigel into the SeedEZ™, please see SeedEZ™ Cell Seeding Protocols.

RECOMMENDATIONS

USE CONICAL-BOTTOM OR V-BOTTOM MULTI-WELL PLATES FOR CELL RECOVERY. These plates allow to easily separate SeedEZ™ substrates from respective cell pellets. After spin, SeedEZ™ substrates are typically seated horizontally and trapped in the "cone" at a vertical distance from the cell pellet which is seated at the bottom of the "cone". This allows easy substrate removal without disturbing the cell pellet.

IF CONICAL- OR V-BOTTOM PLATES ARE UNAVAILABLE IN A DESIRED FORMAT, USE REACTION (PCR) PLATES AND SEAL THEM FOR SPIN. The PCR plates are available in a 24-well, 48-well, 96-well format and higher. As PCR well-diameters may be smaller than those of cell culture plates of the same format, check well diameter before deciding which PCR plate to use. (Note that most PCR plates are made of polypropylene and can be sterilized by steam autoclaving. Ask the plate supplier for recommended parameters. Some suppliers recommend 121° C for 15 minutes at 15 psi.)

Flat-bottom multi-well plates are not recommended.

Flat-bottom plates may not yield a complete cell recovery following spin. The SeedEZ™ removal from respective wells after spin may also trap cells.

Multi-well inserts in a multi-well plate, or integral multi-well insert systems are not recommended.

Inserts typically comprise membranes which pores are too small and in insufficient number for complete cell isolation for most cell types when the SeedEZ™ substrates are seated in the insert.

MATERIALS

- Uncoated or Poly-D-Lysine coated SC-C048 SeedEZ™ substrates comprising cells embedded in GFR Matrigel matrix
- 48-well plate with the above SeedEZ™ substrates seated one per well
- Growth factor containing culture medium; or culture medium and Trypsin neutralizing solution
- Phosphate-Buffered Saline (PBS) 1X without Calcium and Magnesium
- Cell Recovery Solution; BD Biosciences Product No. 354253
- 0.25% Trypsin- 1 mM EDTA; Life Technologies Product No. 25200
- A polyurethane foam ice bucket or pan for seating the multi-well plates in ice
- Disposables: *conical-bottom or V-bottom multi-well plates*, strip caps or silicone rubber sealing mats if PCR plates are used, and micropipette tips (long tip)

EQUIPMENT

Cell isolation from gelled Matrigel in SeedEZ™ substrates seated in the wells of the multiwall plate may be automated and throughput increased using the following equipment:

- A refrigerated centrifuge with a rotor capable of carrying and spinning multi-well plates.

  If unskirted PCR plates are used, semi-skirted of fully skirted PCR plates may be used as adapters, if necessary. Alternatively, use dedicated PCR plate adapters, or removable PCR tube tray holders with lids provided they fit into the swing-bucket rotor used for spinning down the plates.

- A vortexing shaker, a microplate shaker, or an orbital shaker with the speed of no less than 200 RPM capable of holding microtiter plates seated in ice in a polyurethane foam ice pan or bucket for the Cell Recovery Solution treatment.

- A shaking water bath for 37°C Trypsin treatment.

- Sterilization equipment for non-sterile plates and lids, if sterile conditions are used in subsequent cell studies.

  If using steam autoclave for non-sterile polypropylene PCR plates, polypropylene strip caps or silicone caps and mats, first run a test cycle using sterilization parameters you commonly use. Polypropylene is generally considered autoclavable; however, inspect the plates and caps for warping at the end of the cycle. If silicone is used, inspect silicone caps or mats. Ensure that at the end of the cycle, the caps/mats still fit into the plates.

- Vacuum source and a vacuum aspirator for multi-well plates.

  After spin, use automated tools or multi-channel pipettes and adapters to place the SeedEZ™ substrates towards the side of the wells without vacuum. Next, apply suction to lift the substrates and transfer.

  You may also use hand-held vacuum aspirator with a multi-channel adapter; for example, an 8-channel adapter.

  In the event you cannot lift the substrates using suction, check your vacuum source. It may be too weak or the system has leaks. If you cannot aspirate the medium, you will not be able to lift the substrates either.

METHODS

Part I: BD Cell Recovery Solution Treatment

FOR THE CELL RECOVERY SOLUTION TREATMENT PRE-CHILL DISPOSABLES AND USE ICE-COLD PBS, CELL RECOVERY SOLUTION, AND MEDIUM. KEEP THE PLATE ON ICE DURING ALL STEPS.

1. Aspirate and discard medium from SC-C048 SeedEZ™ substrates seated in a 48-well plate.
2. Rinse SeedEZ™ substrates seated in a multi-well plate 2X with cold PBS; aspirate the last rinse.
3. Transfer SeedEZ™ substrates to a conical-bottom multi-well plate.

   For SeedEZ™ SC-C048 substrates, transfer the substrates to a 48-well or a *24-well PRC plate.

   * Some plates may be thick-walled. Ensure that 9.5 mm in diameter SeedEZ™ SC-C048 substrate fits into the well of the 48-well plate. Alternatively, use a 24-well PCR plate.

4. Add BD Cell Recovery Solution enough to submerge the SeedEZ™ substrates; 500 µl recommended.
5. Seal the plate, place in an ice filled pan, and transfer the pan with the plate to orbital shaker set to 150-200 RPM.
6. Shake on ice for at least 2 hours.

7. Transfer the tube to a 4°C centrifuge and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).
8. Put plate on ice and transfer to hood.
9. Push the SeedEZ™ substrates towards the side of the well (be careful not to disturb cell pellets at the bottom of the conical wells). Use pipet tips, but do not use suction.
10. Use suction to transfer the SeedEZ™ substrates to another conical-bottom plate (this plate is used in Part II of the protocol).
11. Aspirate supernatant (leave approximately 50 µl).

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.
12. Re-suspend cell pellets in the approximate 50 µl volume, if cell count (see Step 13) is the objective.

    You may re-suspend in larger volume; however, for accurate cell count from each SeedEZ™ substrate do not dilute too much as you may have too few cells if the seeding density was low (< 10^6 cells/ml) and the cells were post- mitotic, or the cells did not proliferate owing to their phenotype, medium composition or a short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Wash the pellet by gentle resuspension in cold PBS, followed by spin in a 4°C centrifuge at 1000 RCF for 5 minutes. Aspirate supernatant and resuspend cells in an appropriate volume of medium. Combine the cells with the cells from the Trypsin treatment (see Part II) and use for re-plating (if sterile conditions were maintained), or biochemical/ molecular studies; for example, to extract RNA or nuclear proteins.
13. Count cells using methods you normally use.

    The cell count shows effectiveness of cell isolation from Matrigel embedded in the SeedEZ™ using BD Cell Recovery Solution using this protocol.

Part II: 0.25% Trypsin – 1 mM EDTA Treatment

14. Rinse SeedEZ™ substrates seated in a conical-bottom multi-well plate twice with PBS; aspirate the last rinse.
15. Add 0.25% Trypsin-EDTA pre-warmed to 37°C.
16. Seal the plate and transfer to a shaking water bath set to 37°C for 5-10 minutes.

    Shorter trypsinization gives higher cell viability but potentially lower number of recovered cells.
17. Transfer the plate to sterile hood and add at least 2X Trypsin volume in Step 15 in medium to neutralize Trypsin.

    Depending on plate used and volume used in Step 15, you may have to aspirate Trypsin and then add medium.
18. Seal the plate and transfer to centrifuge, balance, and spin at 1000 RCF for 5 minutes (1000 RCF = 1000 x g).
19. After spin down, transfer the plate to hood.
20. Push the SeedEZ™ substrates towards the side of the well (be careful not to disturb cell pellets at the bottom of the conical wells). Use pipet tips, but do not use suction.
21. Use suction to transfer the SeedEZ™ substrates to another plate (see Step 25).

22. Aspirate supernatant (leave approximately 50 µl).

    As cells are dissociated from a 9.5 mm in diameter SeedEZ™ SC-C048 substrate, not a T-75 flask, the cell pellet will be small.

23. Re-suspend cell pellets in the approximate 50 µl volume, if cell count (see Step 24) is the objective.

    You may re-suspend in larger volume; however, for accurate cell count from each SeedEZ™ substrate do not dilute too much as you may have too few cells if the seeding density was low (< 10^6 cells/ml) and the cells were post- mitotic, or the cells did not proliferate owing to their phenotype, medium composition or a short culturing period.

    IF CELL COUNT IS NOT THE OBJECTIVE: Resuspend cells in an appropriate volume of medium. Combine the cells with the cells from the Cell Recovery Solution treatment (see Part I) and use for re-plating (if sterile conditions were maintained), or biochemical/ molecular studies; for example, to extract RNA or nuclear proteins.

24. Count cells using methods you normally use.

    If cells remained embedded in Matrigel in the SeedEZ™ following the Cell Recovery Solution treatment (see Part I), the cell count here shows the effectiveness of cell isolation from Matrigel embedded in the SeedEZ™ using a 2-hour BD Cell Recovery Solution treatment at 4°C, followed by 5-10 minute 0.25% Trypsin-EDTA treatment at 37 °C.

25. To check for completeness of cell recovery, stain and image cells (if any) in the SeedEZ™ substrates in a flat-bottom multi-well plate into which the substrates were transferred to following spin down (see Step 21).

CELL RECOVERY RATE MIGHT BE FURTHER INCREASED USING 4°C TRYPSINIZATION AND SPIN. AT 4°C MATRIGEL REMAINS AT ITS SOL-STATE. THIS FACILITATES CELL RECOVERY IF THE GEL DID NOT FULLY DE-POLIMERIZE USING THE BD BIOSCIENCES CELL RECOVERY SOLUTION TREATMENT. FURTHER, AT 4°C TRYPSIN MAY BE APPLIED LONGER THAN 5-10 MINUTES WITH THE ADDED ADVANTAGE OF BEING GENTLER TO THE CELLS THAN AT 37°C.