Generation of tissue organoids by compaction reaggregation

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Abstract

Cellular reaggregation methods are commonly used to generate tissue organoids for use in biological studies. Using a modified method termed 'compaction reaggregation', it is possible to establish reaggregates of reproducible size from defined input cell numbers with ease and without specialist equipment. Importantly, this method is suitable for the study of tissues that have proved refractory to reaggregation by other methods. With the option of juxtaposing cell populations, this method is useful for studies of tissue organization and structure.

Key words: aggregate; reaggregate; CoROC; organoid; thymus

1 Introduction

Organ reaggregation has been used to study the cellular and molecular requirements for diverse processes in many tissue systems including limb(1), skin(2, 3), thymus(4) and lymph node(5). With most solid tissues, it is possible to promote some cellular aggregation in suspension culture however to promote the degree of aggregation necessary to generate organotypic structures, it is often necessary to encourage cell-cell interaction by bringing the cells into close proximity and for this several strategies have been developed. Using gravity, the hanging drop method encourages aggregation of cells that gather at the base of a suspended droplet of medium. As the process is inefficient, many cells fail to aggregate and resulting aggregates are generally small and of variable size. To encourage larger aggregate formation, centrifugation can be used to generate a cell pellet at the base of a well in a U- or V-bottomed multi-well plate, which aggregates upon subsequent culture(6). This method incorporates

pelleted cell types into the aggregate even if some of those cells are not themselves capable of aggregation such as those of some haematopoietic lineages(5, 6). One further advantage of a centrifugation method is that using iterative cell addition and centrifugation steps it is possible to organize cell types in layers, a useful tool when studying cell-cell interactions. Although highly reproducible when using permissive cells, several tissue types reaggregate poorly or not at all under submerged culture conditions and require aggregation at the gas-liquid interface(7). This can be achieved by generation of a viscous cell slurry, which is then deposited as a standing drop on a filter paper disc floating at the gas-liquid interface where an aggregate can form(4). Although broadly applicable, aggregation in a standing drop is less controlled than in the above-described centrifugation method, and neither cell input numbers nor cell type position can be controlled(7).

A modified aggregation method, termed compaction reaggregation (CoROC), relies upon the generation of a cell pellet and subsequent extrusion as a single mass onto a membrane at the gas-liquid interface. CoROC combines the reproducibility and layering capabilities of the centrifugation method with the broad tissue applicability of aggregation at the gas-liquid interface. With this one method it is possible to investigate developmental processes in tissues that are known to be resistant to submersion reaggregation and for which composition and placement are key.

These instructions are for the generation of CoROCs of fetal thymus utilizing primary fetal thymus tissue as an example of a submersion-sensitive organ containing both aggregating (epithelial) and non-aggregating (haematopoietic) cell types. Though not necessary when using whole dissociated fetal thymus, when reaggregating purified epithelial cells fibroblasts are included to provide factors required for proper epithelial development, as well as either immature thymocytes or lymphocyte precursors. These can be primary fibroblasts such as murine embryonic fibroblasts (MEF), or a fibroblast cell line, such as NIH/3T3. In the example given, NIH/3T3 cells

are included as a separate cell population with which to illustrate the layering technique. A similar strategy to that outlined below has been successfully used for other tissue types including the aorta-gonad-mesonephros region (7, 8) and mammary gland (unpublished data) using tissue-appropriate dissociation methods and growth media. In cases where this has not been attempted before, it is recommended that these are determined for each tissue type using this method as a starting point.

2 Materials

2.1 Preparation of cell suspensions for reaggregation of mouse fetal thymus

1. Dulbecco's phosphate buffered saline solution (DPBS).
2. DPBS, modified, without calcium and magnesium (DPBS-CMF).
3. Solution of 0.025% trypsin with 1mM ethylenediamine tetraacetic acid (EDTA) in DPBS-CMF. Store aliquots at -20°C
4. Enzymatic dissociation mix. Solution of DPBS containing 2 mg/mL hyaluronidase, 0.7 mg/mL collagenase, and 0.05 mg/mL DNase I. Use immediately or store single use aliquots at -20°C.
5. 0.4% trypan blue solution prepared in DPBS-CMF, filtered through 0.45μm filter unit. Store prepared working solution at room temperature.
6. Hemocytometer and coverslip.
7. Wash solution of DPBS-CMF containing 10% fetal calf serum.
8. Tissues or cells for reaggregation.

2.2 Compaction reaggregation at the Gas/Liquid Interface

1. 200μL non-beveled tips (Axygen, Union City, CA)
2. 0.8μm Isopore membrane filters (Millipore, Billerica, MA)
3. Parafilm M (Pechiney Plastic Packaging Company, Chicago, IL; see **Note 1**)
4. Sterile 6 well plate

5. Cell growth medium (CGM): DMEM/F12 supplemented with 10% FCS, 1x non-essential amino acids, 10mM HEPES Buffer, 0.05mM β-mercaptoethanol and 100IU/mL penicillin/streptomycin (all Invitrogen). Store prepared medium at 4°C and use within one week.
6. Flask of NIH/3T3 fibroblasts at 80% confluence. NIH/3T3 are maintained in DMEM containing 10% FCS and 100IU/mL penicillin/streptomycin and passaged when 80% confluent with trypsin/EDTA.
7. Stereomicroscope (see **Note 2**)

3 Methods

3.1 Preparation of cell suspensions for reaggregation

1. Materials for the preparation of a cell suspension are made ready (see **Note 3**).
2. A flask of NIH/3T3 should be harvested by incubation with trypsin/EDTA at 37°C, washed in wash solution then centrifuged at 200xg for five minutes before being resuspended in CGM and placed on ice.
3. Primary fetal thymus tissue is dissected from embryonic day (E) 11.5 to 15.5 embryos under clean conditions and placed in an eppendorf tube containing wash solution at room temperature (see **Notes 4 and 5**).
4. Pre-warm enzymatic dissociation mix to 37°C in heating block for three minutes.
5. The eppendorf tube containing the lobes is centrifuged at 200 x *g* for three minutes to collect the lobes before the supernatant is removed and replaced with pre-warmed enzymatic dissociation mix. As a guide, for E11.5 to E13.5 tissue from fewer than five litters of mice, 0.5mL enzymatic dissociation mix should suffice. For between five and ten litter or when dissociating lobes from older embryos, increase this amount to 1mL. Flick the tube to

resuspend the lobe pellet and incubate at 37°C for 10 minutes in a heating block.

6. Gently triturate the suspension five times, avoiding bubble formation, using a P1000 pipette to aid tissue break-up and release cells (see **Note 6**).
7. Incubate for a further five minutes at 37°C.
8. Triturate the sample ten more times and visually inspect the resultant suspension to determine whether tissue fragments remain. At this point, E11.5 to E13.5 lobes should have dissociated fully and therefore will require no further treatment (proceed to **Step 12**). Should a further digestion be necessary, as would be the case with E14.5 to E15.5 lobes, pre-warm 0.5mL trypsin/EDTA (see **Note 7**).
9. Centrifuge the suspension containing the dissociated cells and remaining tissue fragments at 300 x *g* for five minutes to collect the cells.
10. Remove the supernatant and replaced with pre-warmed trypsin/EDTA.
11. Resuspend the cell pellet by briefly flicking the tube and incubate for three minutes then gently triturate the sample to ensure all cells are released from the fragments.
12. Once satisfactorily dissociated, fill the remaining volume of the eppendorf tube with D-PBS+FCS and spin the single cell suspension for five minutes at 300 x *g* to collect the cells (see **Note 8**).
13. Discard the supernatant containing the enzyme and resuspend the cell pellet in CGM for counting. Due to the small number of cells likely to be isolated from embryo dissections, as a guide, 200μL volume would be a suitable resuspension volume for counting thymi from each litter of E13.5 embryos.
14. Cell types that will be added to the CoROC should be counted using a 1:1 mix of cells to 0.4% trypan blue solution to enable trypan blue-excluding, viable cells to be enumerated.
15. A determination of input cell number and type should be made at this point taking into consideration ensuring that the relative sizes of cells present to

generate reaggregate is neither too small to study nor too large to maintain viability. A typical fetal thymus CoROC contains between 150,000 cells (50,000 thymus cells plus 100,000 NIH/3T3) and 300,000 cells (200,000 fetal thymus cells plus 100,000 NIH/3T3) with the majority of the aggregate size coming from the much larger fibroblast cells. Such a mix would typically form a reaggregate of approximately 400μM to 600μM in diameter, an ideal size for reaggregation in a 200μl tip (see **Notes 9 and 10**).

3.2 CoROC at the gas/liquid interface.

1. Aliquot cells to be included in each CoROC into a fresh tube so that all necessary cells are represented in one pool of cells of not greater than 100μl (see **Note 11**).
2. Draw the suspension containing cells to be reaggregated into a non-bevelled 200μl pipette tip. Set down the pipette with the tip still attached to free up hands for the next stage.
3. Seal the narrow tip aperture with Parafilm as outlined below and shown in Figure 1 (see **Notes 1 and 12**). Parafilm of approximate dimensions 1cm x 5cm is cut and folded twice to make a rectangle measuring 1cm x 1.25cm. Gently press the Parafilm over the narrow tip aperture so that the tip makes an indentation in the Parafilm that can be seen and felt on the reverse side (see **Fig. 1a**). It is important that the tip does not protrude so much as to weaken the Parafilm cover as this would result in leakage under the pressure of centrifugation. While maintaining even pressure on the Parafilm where it covers the aperture, fold the Parafilm using the tip as a pivot so that two sides of the Parafilm now lie against the tip wall on opposite sides with the Parafilm now being 8 ply (see **Fig. 1b**). Press these ends lightly together so that the Parafilm is now firmly attached to the tip (see **Fig. 1c**). Fold the Parafilm once again using the tip as a pivot and then press together the two meeting ends (see **Fig.1d and e**). This should leave 16-ply Parafilm square

that securely encases the tip aperture and that overhangs the tip on one side (see **Fig. 1f**).

4. Transfer the assembly to a 50mL centrifuge tube taking care not to dislodge the Parafilm seal. Centrifuge at 300 x *g* for five minutes so that a cell pellet is formed at the occluded aperture of the tip adjacent to the Parafilm seal.
5. Prepare the culture plate. Add 3mL CGM to a 6 well plate and overlay a membrane disc taking care not to submerge any part of the disc or allow any media to flow over the edges of the disc, which can result in sinking of the assembly and dispersal of the cells to be aggregated. Position so that the floating membrane is in focus under a stereomicroscope (see **Note 2)**.
6. Reattach the tip to a pipette that has been set to a pipette volume of 30μl. It is necessary to attach the tip cautiously and incompletely as the action of reattachment can generate enough pressure within the tip to expel the pellet.
7. Unfold the Parafilm in preparation for removing it while maintaining slight pressure on the Parafilm at the tip end so that the aperture remains occluded until you are ready to proceed with the extrusion.
8. Position the pipette over the membrane and remove the Parafilm.
9. With the tip aperture almost touching the centre of the floating disc, carefully and slowly, push the tip more firmly into position on the pipette so that a firm seal is made (see **Fig.2a**). This action provides enough pressure within the tip to push the cell pellet outwards through the previously occluded narrow end onto the filter paper disc. Should the pressure be insufficient, extrusion can be forced by incrementally winding the volume setting of the pipette down as far as is required to expel the pellet. In either case, a pellet can be extruded onto the filter with minimal CGM (see **Fig. 2b**).
10. Culture the cell pellet at 37°C for 24 hours allowing time for the formation of a solid reaggregate. As the floating disc is susceptible to submersion, and the pellet itself is subject to disturbance, extreme care must be taken when moving the plate containing the floating assembly to the incubator. After

successful reaggregation, the CoROC can be cultured or transplanted for further study.

3.3 Generating cell layers in a CoROC at the gas/liquid interface

1. Cells to be reaggregated in separate layers should be resuspended in CGM so that the cumulative volume of cells to be included is no greater than 100μL. For example, 40μL thymus cells could be reaggregated with equal to or less than 60μL NIH/3T3 cells.
2. Draw the suspension containing the cells required for the first cell layer into a 200μl pipette tip.
3. With the suspension inside, seal the narrow tip aperture with Parafilm as outlined in **Section 3.2** and **Figure 1**.
4. Transfer the assembly to a centrifuge tube taking care not to dislodge the Parafilm seal and centrifuge at 300 x *g* for five minutes so that a cell pellet is formed at the occluded aperture of the tip adjacent to the Parafilm seal.
5. Without removing the tip from the 50mL tube, the suspension containing cells to be included in the second layer of the CoROC are carefully added to the open end of the tip without disturbing the first pellet that has already been established.
6. The assembled tube should be centrifuged once again to produce a cell pellet that lies on top of the first. Further cell additions and centrifugation steps will produce a multi-layered pellet.
7. Continue as **Section 3.3 Step 6**.

4 Notes

1. Although it is possible that other similar products may be used, the properties of Parafilm have proved ideal.

2. Where possible, the area used for reaggregate assembly should be clean to avoid microbial contamination. While this is best achieved using a stereomicroscope-equipped laminar flow hood, the authors typically use a clean area and considered aseptic technique without resulting in culture contamination
3. All solutions used in the preparation of samples and subsequent culture should be sterile.
4. Noon of the day of the vaginal plug was taken as day 0.5.
5. During dissection ensure that hair and dander contamination is eliminated, as this will be deleterious during enzymatic digestion.
6. The shear forces associated with bubbles can damage cells.
7. As with any dissociation procedure, care should be take not to 'over digest' tissue, as this will reduce cell viability. It is important to note that damaged cells tend to adhere to released DNA forming clumps, which may be mistaken for remaining tissue fragments. In the event that this happens, clumps may be incubated with 0.05mg/mL DNase I in wash solution to digest the DNA that holds these clumps together and limit the formation of further clumps.
8. The addition of FCS will inhibit the trypsin activity and thus halt further digestion.
9. As cell size directly influences reaggregate size, it is necessary to empirically determine optimal cell numbers for each tissue or cell type used.
10. The aperture of the tip in which the cells are compacted is critical. Although a wide bore 200μL tip is capable of generating a cell pellet of larger dimensions, the authors have found that this generally results in cell pellets that when extruded are flatter and less reproducible in aggregation efficiency and therefore size. A 10μL tip can be used to generate smaller CoROC however the force applied during extrusion commonly leads to an excess of extruded CGM along with the cells, which makes reaggregation less efficient. Therefore, in order to utilize different tip sizes for the purpose of

generating CoROC of alternate dimensions, it is advisable to optimise this with a particular cell type and number in the first instance.

11. We have found that the greater the cell suspension volume used, the more chance that the pellet will extrude upon Parafilm removal prior to application of pressure. In such cases, the pellet is often expelled too quickly and results in CGM being also expelled, which disturbs the pellet and lowers aggregation efficiency. Therefore, it is advisable to use minimal volumes with less than 50μL being ideal.
12. The folded Parafilm is critical to the success of the compaction process with most generation errors resulting from this stage of the process. Although various methods could be used, the outlined fold method has proved both simple and reliable.

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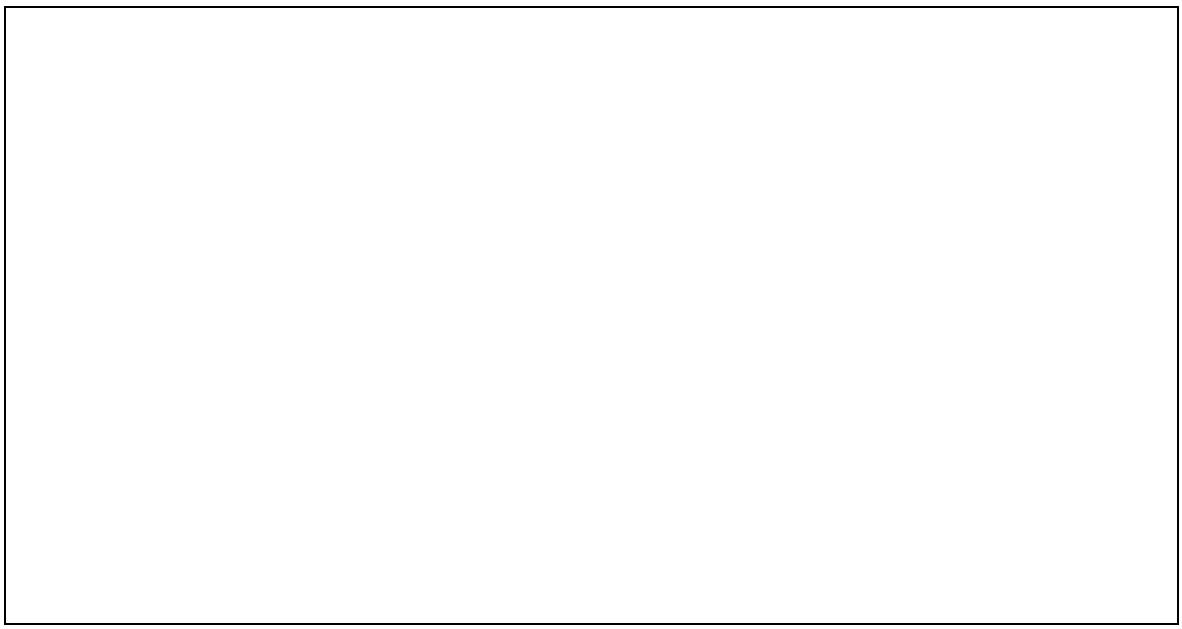

Figure 1

Assembly of the sealed pipette tip in preparation for cell pellet generation. A cell suspension containing 100,000 thymic epithelial cells and 100,000 NIH/3T3 was drawn up into a 200μL pipette tip that was subsequently sealed with Parafilm as shown. This assembly efficiently prevents leakage of the cell suspension prior to and during centrifugation that will result in a cell pellet at the aperture of the tip adjacent to the Parafilm seal.

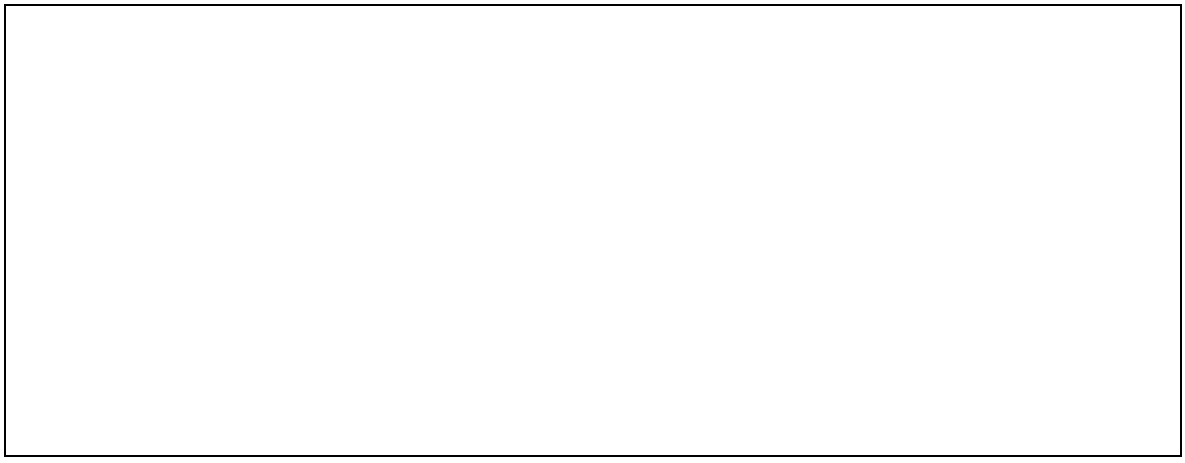

Figure 2

Extrusion of the cell pellet onto an Isopore membrane floating at the gas/medium interface in a 6 well plate. Here, the cells will spontaneously aggregate within 24 hours of culture.