

Laser Capture and Single Cell Genotyping from Frozen Tissue Sections

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Abstract

There is an increasing requirement for genetic analysis of individual cells from tissue sections. This is particularly the case for analysis of tumor cells but is also a requirement for analysis of cells in pancreas from individuals with type 1 diabetes where there is evidence of viral infection or in the analysis of chimerism in pancreas; either post-transplant or as a result of fetomaternal cell transfer.

This protocol describes a strategy to isolate cells using laser microdissection and to run a 17plex PCR to discriminate between cells of haplo-identical origin (i.e., fetal and maternal cells) in pancreas tissue but other robust DNA tests could be used. In short, snap-frozen tissues are cryo-sectioned and mounted onto membrane-coated slides. Target cells are harvested from the tissue sections by laser microdissection and pressure catapulting (LMPC) prior to DNA profiling. This is based on amplification of highly repetitive yet stably inherited loci (short tandem repeats, STR) as well as the amelogenin locus for sex determination and separation of PCR products by capillary electrophoresis.

Keywords: Laser microdissection and pressure catapulting, Pancreatic cells, Diabetes, Tissue

1 Introduction

There has been renewed interest in the study of the pancreas in type 1 diabetes including detailed descriptions of the cells constituting insulinitis [1, 2] as well as studies aimed at detecting evidence of viral infection [3, 4], inflammatory mediators [5], and beta cell stress [6]. Such studies have increasingly been enabled by access to high quality autopsy pancreas tissues through the Network of Donors (nPOD; <http://www.jdrfnpod.org/>). One requirement is the recovery of high quality DNA from individual cells for further DNA investigation, for instance confirmation that a cell, or cells, in a transplanted pancreas originates from the recipient (chimerism) [7] or mother (maternal microchimerism) [8, 9]. We have developed a technique to isolate individual cells from frozen human pancreas for downstream DNA analysis. The DNA test used here generated a profile of 16 polymorphic loci and one sex determining locus [10, 11] but any robust DNA test could be used for DNA analysis, for instance viral DNA tests.

A note of caution; our experiments to date showed that the protocol described here does not work optimally on

1. Formalin fixed paraffin embedded tissue: this is possibly because formalin irreversibly cross-links proteins via the amino groups to preserve the structural integrity of the cells. Isolation of nucleic acids is however impaired by both the paraffin wax and the cross-linking process.
2. Tissue that has been pretested using fluorescence in situ hybridization (FISH) to stain specific DNA sequences.

Further optimisation is required in both these situations.

2 Materials

2.1 Materials for Tissue Preparation

1. Cryostat.
2. Poly ethylene terephthalate (PET) membrane slide.
3. UV light source (254 nm) (i.e., in a tissue culture hood or a DNA cross-linker).
4. 100 % acetone.

2.2 Materials for DAPI Staining

1. 10× phosphate-buffered saline (PBS, pH 7.3): dissolve 2.586 g potassium dihydrogen phosphate (KH_2PO_4), 29.01 g disodium hydrogen phosphate (Na_2HPO_4), and 90.06 g sodium chloride (NaCl) in 900 mL Milli-Q-water. Use a magnet stirrer to dissolve the salts. When all salts are dissolved, top up solution to 1 L and store the solution in a glass flask at RT for up to 6 months.
2. 1× PBS: dilute from 10× PBS buffer, i.e., 100 μL 10× PBS plus 900 μL Milli-Q water (*see Note 1*).
3. Ethanol wash solutions: prepare v/v dilutions of 70, 85, and 100 % using 100 % ethanol in purified water.
4. VECTASHIELD mounting medium with DAPI, H-1200 (Vector Laboratories, UK).
5. Coverslips (22 × 22 mm).
6. Coplin jars.

2.3 Materials for LMPC

1. 1× PBS.
2. PCR-clean tubes (200 μL and 1.5 mL).
3. Plastic centrifugation tubes (50 and 15 mL).
4. Dedicated pipette set and filter pipette tips (20, 200, 1000 μL).
5. PCR-grade water.
6. Mini Centrifuge.

2.4 Materials for Cell Lysis

1. 5× Lysis Buffer: 10 mM Tris-HCl, 6 mM Ca(OAc)₂: Dissolve 1.7618 g of Ca(OAc)₂ per 10 mL of nuclease-free water to obtain a 1 M calcium acetate stock solution. Mix 6 μL of 1 M calcium acetate stock solution and 10 μL 1 M Tris-HCl (pH 7.4) with 984 μL of nuclease-free water and pulse vortex for 15 s. Aliquot the 5× Lysis Buffer and store at −20 °C.
2. Proteinase K storage buffer: 10 mM Tris-HCl, 6 mM Ca(OAc)₂, glycerol 40 vol.%: mix 6 μL of 1 M calcium acetate stock solution, 10 μL 1 M Tris-HCl, 400 μL glycerol and 584 μL Milli-Q water.
3. 5× Lysis Enzyme: dissolve 20 mg proteinase K in 1 mL of the Proteinase K storage buffer, mix well, aliquot and store at −20 °C.

2.5 Materials for DNA Profiling

1. PCR-clean tubes (200 μL and 1.5 mL).
2. Filter pipette tips (10, 200, 1000 μL).
3. Milli-Q water.
4. Centrifuge for a short spin at max 2000 × *g*.
5. PowerPlex[®] 17 ESX System Kit (cat. no. DC6721, Promega, *see Note 2*): 2800 M Control DNA (cat. no. DD710A), CC5 Internal Lane Standard500 (cat. no. DG152A), PowerPlex[®] 17 10× Primer Pair Mix (cat. no. DK149B), PowerPlex[®] ESX 17 Allelic Ladder Mix (DG150B), PowerPlex[®] ESX 5× Master Mix (DP102A), Water, Amplification Grade (DW099A).
6. Matrix Standards (*see Note 3*).
7. Thermo cycler.
8. Capillary analyzer (e.g., 3730 DNA Analyzer).
9. 96-well plates.
10. Sealing foil for 96-well plates.

3 Methods

3.1 Tissue Preparation

1. Treat PET membrane slides under a UV light source at 254 nm for 30 min (*see Note 4*).
2. Section snap-frozen tissue blocks at 5 μm and mount onto PET membrane slides. This requires an experienced operator and we recommend use of a tissue sectioning facility.
3. Air-dry at room temperature for minimum 2 h (can be up to 8 h).
4. Fix in acetone for 10 min at room temperature.
5. Air-dry and store at −80 °C.

3.2 Tissue Staining

1. Remove slides from the $-80\text{ }^{\circ}\text{C}$ freezer and thaw at room temperature.
2. Rehydrate tissues in an ethanol series for 5 min each (100, 85, and 70 %) in Coplin jars.
3. Wash slides in $1\times$ PBS solution for 5 min with gentle agitation at room temperature.
4. Add one droplet of mounting medium containing DAPI and cover section with coverslip (*see Note 5*).
5. Screen the tissue to pre-select areas for subsequent tissue sampling (*see Note 6*).
6. To remove the coverslip, place the slide vertically in a 50 mL tube containing $1\times$ PBS and wait for approx. 15 min for the coverslip to slide off (*see Note 7*).
7. Wash in fresh $1\times$ PBS for another 5 min by tilting the tube gently to remove mounting medium from the tissue surface.
8. Dehydrate the tissue in an ethanol series (70, 85, 100 %) and leave it in 100 % ethanol until LMPC sampling or air-dry the sample and store at $-80\text{ }^{\circ}\text{C}$.

3.3 Microdissection into Caps of PCR Tubes

To perform laser microdissection you either need to be already familiar with the technique and the system available or alternatively to seek the assistance of an experienced collaborator. Perform the microdissection in a room physically separated from PCR amplification areas. Do not enter this area with amplified DNA. The procedure described here uses the Observer Z1 (Zeiss) laser microdissection device equipped with a RoboMover that holds the PCR tube and places it above target cells to allow cell capture upon pressure catapulting. Switch on the system and let it perform initial calibration. Visually inspect the membrane slides before microdissection for blisters as these may interfere with the procedure (*see Note 8*).

1. Prepare ready-to-use lysis buffer by adding $2.0\text{ }\mu\text{L}$ of $5\times$ Lysis Buffer and $2.0\text{ }\mu\text{L}$ of $5\times$ Lysis Enzyme to $6.0\text{ }\mu\text{L}$ of PCR-grade water for every sample to be processed (*see Note 9*). Mix by pipetting, short-spin to collect all liquid at the bottom of the tube and place on ice.
2. Remove the slide from 100 % ethanol and let air-dry for a few minutes (*see Note 10*). Load the slide into the microscope stage and re-locate areas of interest as described previously (*see step 5* of Sect. 3.2, *Note 6*).
3. Load $10\text{ }\mu\text{L}$ of the ready-to-use lysis buffer into the cap of a $200\text{ }\mu\text{L}$ PCR-tube and mount it directly above the sample to be harvested (*see Note 11*).

4. Harvest the first sample by LMPC (*see Note 12*), carefully recover the PCR-tube from the microscope and close the PCR-tube. Immediately centrifuge the tube to collect the sample in the bottom of the tube and place the sample on ice.
5. Harvest the remaining samples including negative controls (*see Note 13*). For positive controls add 9 μL of ready-to-use lysis buffer to the cap of empty PCR tubes.
Directly proceed to DNA typing.

3.4 DNA Typing Using PowerPlex 17 ESX System (Promega)

1. Again short-spin the sample tubes and incubate the samples at 75 °C in a thermal cycler for 5 min followed by an enzyme inactivation step at 95 °C for 2 min (*see Note 14*).
2. Thaw the PowerPlex[®] ESX 5 \times Master Mix and the PowerPlex[®] 17 10 \times Primer Pair Mix on the bench. When the reagents are completely thawed short-spin both vials for 1 s to collect all liquid at the bottom (*see Note 15*). Vortex both vials at full speed for 15 s and again short-spin for 1 s. Place the vials on ice.
3. Prepare an amplification master mix by adding 4.0 μL of ESX 5 \times Master Mix and 2.0 μL of 10 \times Primer Pair Mix to 4.0 μL of PCR grade water for every sample to be processed (*see Notes 16 and 17*), mix the solution by pipetting, short-spin and place on ice.
4. Prepare a DNA control solution using the DNA supplied with the kit or your own and dilute it to maximum 500 pg/ μL (*see Note 18*). Add 1.0 μL of the diluted control DNA to the tube containing 9.0 μL lysis mix.
5. Add 10.0 μL of the amplification master mix to every sample and mix by flicking (*see Note 19*). Short-spin the tubes and place on ice.
6. Denature the samples in a thermal cycler at 96 °C for 2 min and amplify for 30 cycles as follows: Denaturation at 94 °C for 30 s followed by an annealing step at 59 °C for 2 min and an elongation step at 72 °C for 1 min 30 s. For final elongation incubate at 60 °C for 45 min.
7. Store the sample at 4 °C overnight or at -20 °C if not forwarded to post-amplification treatment within the following 24 h.

3.5 Post- amplification Treatment

1. Completely thaw the CC5 Internal Lane Standard 500, short-spin for 1 s and vortex at full speed for 15 s. Again short-spin for 1 s to collect liquid at the bottom of the tube.
2. Thaw amplified samples on the bench, mix by flicking a few times or by pipetting and short-spin.
3. Transfer the total volume of samples to a 96-well plate (*see Note 20*).

4. Load an additional well with 19 μL and add 1.0 μL of PowerPlex[®] ESX 17 Allelic Ladder Mix.
5. Add 0.5 μL of CC5 Internal Lane Standard 500 to all samples and the ladder. Fill up the remaining empty wells with water (*see Note 21*).
6. Cover the 96-well plate with a sealing foil, short-spin up to 1300 rpm and place on ice.
7. Denature the samples in a thermal cycler at 95 °C for 3 min and immediately chill the samples on ice. Directly proceed with capillary electrophoresis.

3.6 Analyze the Samples on any System Capable of Capillary Electrophoresis

To operate capillary electrophoresis instruments you either need to be already familiar with the technique and the system at hand or you need to seek the assistance of an experienced collaborator (e.g., core facility).

4 Notes

1. Rinse the 100 mL measuring cylinder three times with ultra-pure water and add it to the 1 L graduated flask before filling up to 1 L. The resulting 1 \times PBS will be pH 7.3 and does not need further adjustment.
2. The kit comes with two sealed bags. The pre-amplification components box contains the PCR master mix, primer pairs and the control DNA. The post-amplification components contain the internal standard for capillary electrophoresis. The allelic ladder mix is shipped in an extra sealed bag. Upon arrival transfer the allelic ladder mix to the post-amplification component box and store the boxes physically separated in two different areas. It is recommended that the amplified products do not go back to the pre-amplification area. We also recommend using different freezers for storage.
3. Matrix standards are initially required to calibrate the capillary analyzer. The matrix contains all dyes used in the kit and is used to compensate for cross talk. Match the matrix with the capillary electrophoresis system you use:
 PowerPlex[®] Matrix Standards, 310: ABI PRISM[®] 310 Genetic Analyzer.
 PowerPlex[®] Matrix Standards, 3100/3130: ABI PRISM[®] 3100 and 3100-Avant Genetic Analyzers and Applied Biosystems[®] 3130 and 3130xl Genetic Analyzers.
4. Irradiation with UV causes the membrane to become more hydrophilic. This will result in a stronger attachment of the tissue section making it less likely to detach.

5. One droplet of mounting medium is enough to cover a section area of 22×22 mm. Check for equal distribution of the medium across the section. Avoid air bubbles as tissue areas will not be sufficiently labeled with DAPI. Use larger coverslips and volumes of mounting medium if sectioned tissues cannot be covered with a 22×22 mm coverslip.
6. When screening for candidate tissue areas in the DAPI channel keep the exposure to a minimum as elongated exposure can cause DNA degradation in single cells. Once candidate areas are identified take images of the respective region or note the coordinates in order to re-locate the area. This is important because for sampling the coverslip needs to be removed and fluorescence staining bleaches quickly and important orientation may be lost. Furthermore, identify structures within your sample that will serve as reference coordinates (e.g., structures like blood vessels). This will allow you to re-locate candidate tissue areas in case a shift occurs between identifying these areas and harvesting tissue samples. Coverslips slide off easily without any agitation. This works well with slides that were covered with a coverslip up to 3 days stored in the fridge (horizontally). For longer storage of sections, we recommend sealing the coverslip with rubber gum to avoid evaporation that would cause the mounting medium to increase its viscosity which, in turn, would make it more difficult for the coverslip to slide off.
7. If the membrane of the slide shows blisters, open them at the very top by disrupting the membrane with a laser shot into the membrane. The blisters will collapse.
8. Prepare enough ready-to-use mix to run all your samples including negative and positive controls. For example, prepare 200 μL for ten samples and four controls (two negative and two positive), thus allowing for additional solution. Bear in mind that you will need to compensate for pipetting error as well as for additional samples. The latter is due to the recovering process where you might need to discard PCR-tubes (*see Note 11*).
9. If the slide was stored at -80 °C, thaw it and place it again in 100 % ethanol. Let it air-dry and proceed with the protocol.
10. It is crucial to place the 10 μL lysis mix in the middle of the cap (having an “inner ring” structure) so that it forms a nice droplet. This setting (where the recovering droplet can be placed close to the microdissection target) yields highest recovery rates. To avoid the droplet tilting out of place attach it to the rim of the cap. In that situation, 10 μL are not sufficient to cover the whole cap. Cells that are microdissected to PCR plastic might escape analysis as DNA may stick to the plastic.

If the droplet tilts out of place discard the tube and load a new one. Take this into consideration when preparing the ready-to-use lysis mix (*see Note 9*).

11. We recommend checking the PCR-cap for microdissected samples. However, some samples escape this quality control (we only forward PCR-caps containing samples) as they quickly float to the side of the droplet and cannot be detected.
12. We recommend microdissecting membrane containing no sample (e.g., membrane outside the tissue section).
13. This step can be elongated up to 2 h and may vary due to parameter such as type of tissue and thickness of tissue section.
14. Check the PowerPlex[®] ESX 5× Master Mix for precipitate and incubate at 37 °C for a short time in order to dissolve it.
15. The obtained amplification mix is still double concentrated. This is because the amplification master mix needs to be added to the lysed samples at a 1:1 ratio which then yields the final ready-to-use concentration.
16. Prepare enough master mix to amplify all samples, e.g., make sufficient mix for ten samples and when amplifying 8.
17. Aliquot the ready-to-use control DNA. The used aliquot can be stored in the fridge at 4 °C. Store the other aliquots at –20 °C for up to 6 months. Once thawed keep in the fridge.
18. Add the amplification master mix to the tube wall. Do not mix by pipetting as this could cause loss of template DNA.
19. When running samples on the 48-capillary 3730 DNA Analyzer, be sure to add the mix to the correct wells: Load all wells of either even-numbered or odd-numbered columns (for less than 48 reactions including all samples and one well with allelic ladder). For more than 48 reactions also load the other columns.
20. This ensures that no capillary runs dry.
21. If capillary electrophoresis cannot be done directly after the post-amplification treatment, store the samples at –20 °C and perform denaturation (**steps 6 and 7** of Sect. 3.5) again before proceeding to capillary electrophoresis.

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