Video Article

Improved Protocol For Laser Microdissection Of Human Pancreatic Islets From Surgical Specimens

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Abstract

Laser microdissection (LMD) is a technique that allows the recovery of selected cells and tissues from minute amounts of parenchyma [1,2]. The dissected cells can be used for a variety of investigations, such as transcriptomic or proteomic studies, DNA assessment or chromosomal analysis [2,3]. An especially challenging application of LMD is transcriptome analysis, which, due to the lability of RNA [4], can be particularly prominent when cells are dissected from tissues that are rich of RNases, such as the pancreas. A microdissection protocol that enables fast identification and collection of target cells is essential in this setting in order to shorten the tissue handling time and, consequently, to ensure RNA preservation.

Here we describe a protocol for acquiring human pancreatic beta cells from surgical specimens to be used for transcriptomic studies [5]. Small pieces of pancreas of about 0.5-1 cm^3 were cut from the healthy appearing margins of resected pancreas specimens, embedded in Tissue-Tek O.C.T. Compound, immediately frozen in chilled 2-Methylbutane, and stored at -80 °C until sectioning. Forty serial sections of 10 µm thickness were cut on a cryostat under a -20 °C setting, transferred individually to glass slides, dried inside the cryostat for 1-2 min, and stored at -80 °C.

Immediately before the laser microdissection procedure, sections were fixed in ice cold, freshly prepared 70% ethanol for 30 sec, washed by 5-6 dips in ice cold DEPC-treated water, and dehydrated by two one-minute incubations in ice cold 100% ethanol followed by xylene (which is used for tissue dehydration) for 4 min; tissue sections were then air-dried afterwards for 3-5 min. Importantly, all steps, except the incubation in xylene, were performed using ice-cold reagents - a modification over a previously described protocol [6]. utilization of ice cold reagents resulted in a pronounced increase of the intrinsic autofluorescence of beta cells, and facilitated their recognition. For microdissection, four sections were dehydrated each time: two were placed into a foil-wrapped 50 ml tube, to protect the tissue from moisture and bleaching; the remaining two were immediately microdissected. This procedure was performed using a PALM MicroBeam instrument (Zeiss) employing the Auto Laser Pressure Catapulting (AutoLPC) mode. The completion of beta cell/islet dissection from four cryosections required no longer than 40-60 min. Cells were collected into one AdhesiveCap and lysed with 10 µl lysis buffer. Each single RNA specimen for transcriptomic analysis was obtained by combining 10 cell microdissected samples, followed by RNA extraction using the Pico Pure RNA Isolation Kit (Arcturus). This protocol improves the intrinsic autofluorescence of human beta cells, thus facilitating their rapid and accurate recognition and collection. Further improvement of this procedure could enable the dissection of phenotypically different beta cells, with possible implications for better understanding the changes associated with type 2 diabetes.

Video Link

The video component of this article can be found at https://www.jove.com/video/50231/

Protocol

1. Freezing of Human Pancreatic Tissue

1. Remove adipose tissue, blood vessels, nerves and non-parenchymal tissue with a scalpel and tweezers, and cut the pancreatic tissue into pieces (~0.5-1 cm cubes).
2. Place one pancreatic tissue piece in the center of a Cryomold, and cover it completely with cold Tissue-Tek O.C.T. Compound. Put the Cryomold into a jar whose bottom is covered with pre-cooled 2-Methylbutane and snap-freeze in liquid nitrogen. Store the frozen sample at -80 °C.

2. Preparation of Frozen Sections

1. Wear gloves throughout all steps to avoid denaturation of RNA.
2. Clean the cryostat knife, specimen holder and paintbrush with cold 100% ethanol and RNase Away. Place the frozen tissue inside the cryostat.
3. Wait until the tissue, knife and brush reach -20 °C. Label the SuperFrost Plus slides and pre-cool them inside the cryostat chamber while waiting for the tissue to reach -20 °C.
4. Apply the Tissue-Tek O.C.T. Compound on the specimen holder and place the frozen tissue on top.
5. Once the Tissue-Tek O.C.T. Compound is frozen trim the cryoblock and remove any excess of Tissue-Tek O.C.T. Compound with a razor blade.
6. Cut a total of 40 consecutive 10 µm slices from the pancreatic tissue block. Additionally we recommend cutting a first and middle slice to be saved for overview drawings of the pancreatic section that can facilitate the identification of islets within the slices during the microdissection process.
7. Transfer the sections from the knife blade to the center of a SuperFrost Plus slide by touching the backside of the slide with your finger (covered with a glove) to warm up only the region to which the section shall adhere.
8. Dry the sections 1-2 min inside the cryostat at -20 °C, after which you put them into a slide box placed on dry ice. Store the sections at -80 °C.
9. If necessary, clean the knife blade with paper towels and cold 100% ethanol.

3. Dehydration of Frozen Sections

1. Prepare the Reagents: pour 30 ml freshly prepared 70% ethanol, 30 ml DEPC-treated water, 2x30 ml 100% ethanol and 30 ml xylene into 50 ml Cellstar tubes (Falcons); chill and keep all reagents (except xylene) on ice.
2. Clean the working space under the hood with 100% ethanol and RNaseZAP.
3. Process two cryosections as follows: fix in ice cold 70% ethanol for 30 sec, wash with 5-6 quick dips in ice cold DEPC-treated water, dehydrate twice for 1 min in ice cold 100% ethanol, incubate for 4 min in xylene at room temperature (25 °C), and air dry for 3-5 min. Repeat this step with another pair of cryosections.
4. Insert two dried cryosections back to back into a 50 ml Cellstar tube wrapped with aluminium foil to protect them from light and moisture.

4. Laser Microdissection of Beta-cells with PALM MicroBeam, Zeiss

1. Clean the microscope with RNaseZAP and mount the Adhesive Cap in the RoboMover.
2. Set the following settings: Filter set 09 (excitation: 450-490 nm, emission > 515 nm), AutoLPC mode, 50% laser energy, 65% laser focus, 100% laser speed, 5 µm distance between AutoLPC shots, 6 µm distance to line, working height: -10,100.
3. Insert one slide into the stage.
4. Locate the autofluorescent beta cells under microscopic visualisation (5x or 10x lens).
5. Switch to the 40x lens, select the beta cells with the "Freehand" selection tool and microdissect them using the laser.
6. Capture the tissue of four dehydrated cryosections into one AdhesiveCap.
   Comment 1: the time to dissect the beta cells of one dehydrated cryosection should not be longer than 10-20 min to avoid rehydration of the tissue sections which would result in RNA degradation.
   Comment 2: verify successful microdissection process by visual inspection after moving the stage to the checkpoint.

5. Lysis of Microdissected Beta Cell Enriched Tissue

1. Remove the AdhesiveCap from the RoboMover.
2. Pipet 10 µl extraction buffer (XB, PicoPure RNA Isolation Kit, Arcturus) into the lid of the AdhesiveCap and incubate it upside down at 42 °C for 30 min.
3. Spin down at 10,000 x g and put the lysate on dry ice. Store it at -80 °C until the RNA extraction is performed.
4. Repeat steps 3.) to 5.) with all other sections.

6. RNA Extraction

1. Combine all ten 10 µl lysates

2. Proceed with the RNA isolation by working at room temperature according to the protocol of the PicoPure RNA Isolation Kit, Arcturus, using a 1:1 ratio Extraction Buffer (XB) to 70% Ethanol (including DNase Treatment).

7. RNA Analysis

1. Use 1 µl RNA for analysis of quality and quantity using an Agilent 2100 Bioanalyser (PicoChip). Quantitative evaluation is done in reference to a standard RNA loaded in parallel on the chip.

Representative Results

As shown in **Figure 1**, the modified dehydrating protocol led to an improvement of beta cells autofluorescence compared to the previous published protocol [6]. Applying the described protocol, each of 39 surgical pancreatic specimens was used to generate 40 serial cryosections for an average of 31'544'704 µm^3 tissue/ pancreatic specimen (range: 8'742'390 - 81'522'153 µm^3) as shown in **Table 1**. This represents the volume of about 18 pancreatic islets of 150 µm diameter, or of 35,000 β-cells. The tissue originated from an average of 38 different islets/pancreatic specimen (range: 19 - 80 islets), each of which was typically observed in two or more consecutive sections. The large variability in the yield of islets among different specimens reflects the heterogeneity of the tissue source, either pancreatic head or tail, as well as the progressive improvement of the operators in the detection of the islets within the 10 min time devoted to the inspection of each section.

Total RNA from each microdissected and lysed tissue was purified with the Arcturus PicoPure Frozen RNA Isolation Kit of Applied Biosystems and eluted in 11 µl, according to the protocol of the manufacturer. Subsequently 1 µl RNA of each sample was analyzed by the Agilent 2100 Bioanalyser. We obtained on average 42 ng RNA/specimen (range: 5 - 229 ng/specimen) with a mean RNA integrity number (RIN) of 5.8 (range: 3.4 - 7.2). **Figure 2** shows an example of the RNA analysis. Whichever the RIN value all analyzed RNA samples were successfully used for transcriptomic studies. We did not find a direct relationship between the amount of microdissected tissue and the RNA yield. Differences in the time interval between the harvesting by the surgeons and the freezing of the pancreatic specimens after its examination and release by the pathologist could account in part for the variable quality and quantity of recovered RNA. Other key factors to consider are the laser energy applied for microdissection, with the lower energy yielding the higher RNA integrity, as well as the laser focus and the distance of the LPC points. The focus is ideally set when a slight "impact" of the laser on the glass surface of the slide, forming a small black dot, can be seen. In our hands the best results are achieved with a laser energy of 50%, a laser focus of 65%, albeit adapted to compensate for differences in the thickness of the sections, and a distance of LPC points of 5 µm. Additionally, the RNA quantity can be optimized by keeping the working height of the RoboMover, *i.e.* the distance between the adhesive cap and the section, as low as possible to avoid loss of microdissected tissue during the capturing process. In our set up the working height is at -10,100 arbitrary PALM units.

specimen	number of islets/ specimen	collected tissue volume / specimen [µm^3]	RIN	concentration [ng/µl]	total RNA [ng]
DP002	34	24'947'696	5.2	1.149	10.34
DP003	30	41'141'488	5.5	1.677	15.09
DP005	23	27'024'264	3.5	8.259	66.07
DP006	25	45'900'848	3.4	7.399	59.19
DP007	30	23'936'048	6.5	0.595	4.76
DP008	22	68'248'432	6.2	1.559	14.03
DP010	34	33'156'752	5.6	1.750	19.25
DP011	29	31'339'152	6.0	1.365	15.02
DP012	34	57'594'360	6.3	3.025	33.28
DP017	35	28'212'256	6.0	1.292	14.21
DP030	35	48'007'530	5.6	2.180	23.98
DP013	36	34'371'045	6.9	1.350	14.85
DP014	35	21'360'060	7.2	1.270	13.97
DP015	33	20'923'488	4.1	4.000	44.00
DP019	40	35'431'704	7.0	1.670	18.37
DP025	19	15'329'844	6.6	0.496	5.46
DP034	19	81'522'153	6.4	4.260	46.86
DP039	32	19'074'410	6.5	1.270	13.97
DP040	44	31'565'630	5.4	2.300	25.30
DP042	52	40'333'840	7.0	2.510	27.61
DP021	65	33'996'260	6.3	2.830	31.13

DP023	19	19'513'310	5.6	8.250	90.75
DP024	32	24'125'140	6.3	20.780	228.58
DP038	23	18'306'040	6.6	11.700	128.70
DP045	35	36'345'620	6.3	1.100	12.10
DP033	42	26'078'550	6.5	15.380	169.18
DP022	49	33'535'460	6.8	7.590	83.49
DP041	56	34'899'830	5.7	3.350	36.85
DP049	38	18'521'230	6.8	1.020	11.22
DP028	32	16'410'670	5.6	1.720	18.92
DP056	36	19'737'580	5.7	1.130	12.43
DP059	52	23'376'180	3.6	7.880	86.68
DP047	41	20'658'370	6.4	1.040	11.44
DP036	23	8'742'390	3.5	0.950	10.45
DP027	42	29'150'480	5.1	3.410	37.51
DP046	54	16'800'070	5.3	8.210	90.31
DP055	57	32'340'270	6.2	1.280	14.08
DP064	74	41'896'460	6.5	2.440	26.84
DP067	80	46'388'540	6.3	5.200	57.20
average	**38**	**31'544'704**	**5.8**	**3.965**	**42.14**
		number of islets / specimen	**collected tissue volume / specimen [μm^3]**	**RIN**	**total RNA [ng]**
	range	19 - 80	8'742'390 - 81'522'153	3.4 - 7.2	4.76 - 228.58
	average	38	31'544'704	5.8	42.14

Table 1. Overview of the amount of microdissected tissue and the quality and quantity of the extracted RNA. The specimens are listed chronologically. The collected tissue volume is calculated as follows: thickness of cryosection (10 µm) x microdissected area [μm^2]. 1 µl RNA of each sample was analyzed on the Agilent 2100 Bioanalyser PicoChip.

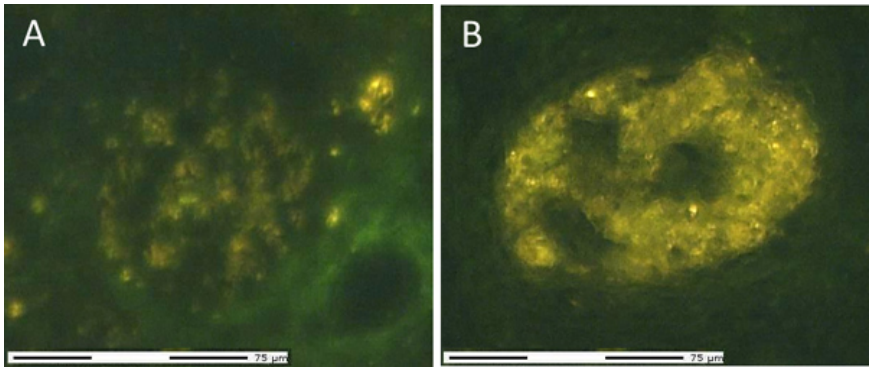

Figure 1. Intrinsic autofluorescence of human beta cells. Autofluorescence of beta cells after dehydration of pancreatic cryosections with reagents which were either kept at room temperature (A), or ice-cold (B) (excitation 450 - 490 nm, emission > 515 nm; 400 x magnification). Beta cells appear yellow, whereas pancreatic ducts, vessels and collagen display a green color. Scale bar: 75 µm.

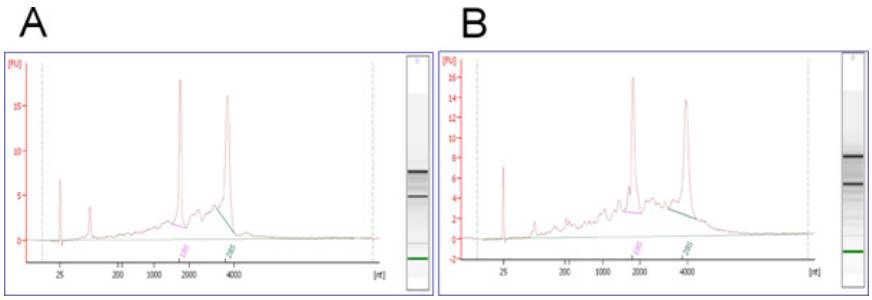

Figure 2. Quality and quantity of extracted islet RNA. Profile of a human islet RNA sample obtained with this LMD protocol (B) compared to a standard RNA sample (A). 1 µl RNA of each sample was applied on a PicoChip and analyzed for quantity and quality with the Agilent 2100 Bioanalyser. (A): RIN (RNA integrity number) 7.7 [rRNA Ratio (28S/18S): 1.3)]; (B): RIN 7.2 [rRNA Ratio (28S/18S): 1.1)]. The concentration of the islet RNA was 1.3 ng / µl, for a total amount of 10.3 ng from 21'360'060 μm^3 pancreatic tissue. Click here to view larger figure.

Discussion

We describe a reliable approach for the laser microdissection (LMD) of human islets from surgical pancreatic specimen. Provided that a LMD microscope is available, this procedure could be implemented at any research institution performing partial pancreatectomies, thereby increasing access to human islet material from both non-diabetic and type 2 diabetic subjects. This is especially relevant given the paucity of pancreata offered for islet isolation. Favourable aspects of LMD compared to islet isolation by collagenase digestion are the reduced time between explantation of the tissue and processing of the islets for RNA extraction [7], the enrichment of beta cells [7], as well as the avoidance of harsh mechanical and enzymatic manipulations [8,9,10], which could alter the gene expression profile. In the case of partially pancreatectomized patients more clinical and metabolic information is typically available than in the case of organ donors. On the other hand, isolation of islets by LMD yields less RNA with lower RIN values compared to those observed after collagenase digestion and does not provide living islets for functional studies. Moreover, the pancreatic disease leading to surgery may affect the islet expression profile. A balanced evaluation of these pros and cons will be achievable through comparative studies on a large number of islet isolations carried out by collagenase digestion and LMD either from organ donors or partially pancreatectomized patients, such as those on-going in the multicenter Innovative Medicine Initiative for Diabetes (IMIDIA) with the goal of "Improving beta-cell function and identification of diagnostic biomarkers for treatment monitoring in Diabetes" (http://www.imidia.org).

Disclosures

No conflicts of interest declared.

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