Human bile duct biopsies: dissociation and single-cell RNA sequencing

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Background

We previously performed several pilot experiments of dissociating fresh and cryopreserved biopsies of bile duct from PSC patients. We observed higher cell count and viability of fresh tissue compared to cryopreserved, however the downstream flow cytometry analysis was inconclusive:

- large population of cells remained uncharacterized since the FC panel was limited to just a few markers – EPCAM, CD45, CD31 (and DAPI as a viability marker)
- had no chance to try CK-7 marker (it requires an intracellular stain)
- detected almost no epithelial cells, and had an issue with compensation since a double positive EPCAM+CD45+ population appeared

Therefore, there still a couple of questions remain:

- are epithelial cells truly absent in bile duct single cell suspensions? If so, is it due to disease, or due to the dissociation protocol, or due to flow cytometry measurement? It is known that enzymatic dissociation causes stress to epithelial cells, so if these cells already damaged, they might not survive the collagenase incubation or nozzle pressure during flow cytometry measurement. If we optimize the dissociation protocol so that epithelial cells are spared the collagenase exposure, would that improve their survival?
- do we capture cholangiocytes? If so, to what degree?
- which immune cells are present in the mix?

Aim

In order to answer all the questions above, we aimed to perform a pilot single-cell RNAseq experiment where we dissociate bile duct biopsies into single cells using either a one-step collagenase protocol or a two-step (EDTA-collagenase) protocol.[1] In short, a two-step protocol directs that whole biopsies are first treated with EDTA for 15 min at 37°C to separate epithelial layer, then the remaining biopsy chunks are dissociated by incubation with collagenase IV for 25 min at 37°C.

We aimed to run this experiment of fresh and cryopreserved bile duct biopsies side-by-side, but unfortunately fresh tissue was not available on that day.

Main conclusion

We can obtain viable single cells from cryopreserved biopsies, but the biopsies must be free of bile.

(1) Uniken Venema, W. T. C.; Ramírez-Sánchez, A. D.; Bigaeva, E.; Withoff, S.; Jonkers, I.; McIntyre, R. E.; Ghouraba, M.; Raine, T.; Weersma, R. K.; Franke, L.; Festen, E. A. M.; van der Wijst, M. G. P. Gut Mucosa Dissociation Protocols Influence Cell Type Proportions and Single-Cell Gene Expression Levels. *Sci. Rep.* **2022**, *12* (1), 9897. https://doi.org/10.1038/s41598-022-13812-y.

Samples

We used 2 cryovials that we received from VUMC (photo 1):

Sample 1: p-0002 from 21-02-2023
Sample 2: p-0004 from 28-03-2023

Photo 1. Used cryovials with bile duct biopsies

Dissociation

Both cryovials were thawed according to the standard procedure. The biopsies from p-0002 looked as expected, while biopsies from p-0004 contained brown residue which is likely to be crystallized bile (photo 2).

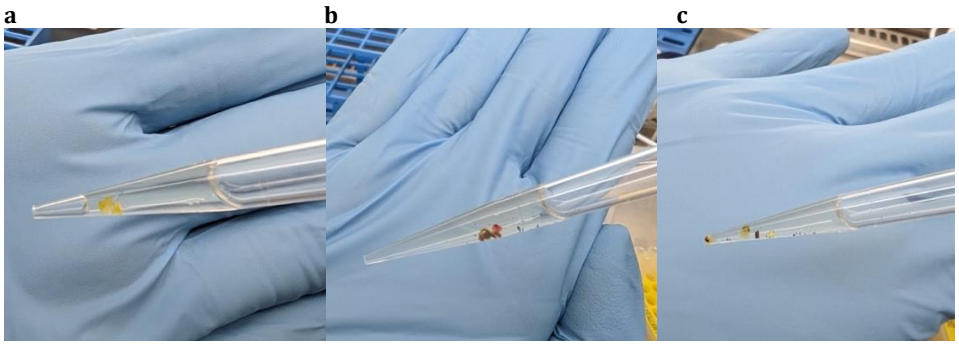


Photo 2. Biopsies from p-0002 (a) and p-0004 (b and c)

Sample 1 was dissociated into single cells according to one-step collagenase protocol (collagenase IV for 25 min at 37°C). Sample 2 was subjected to a two-step protocol, where biopsies were first incubated with EDTA for 15 min at 37°C (sample 2a), and the biopsy chunks were transferred into a separate tube and dissociated by collagenase (sample 2b).

Video snapshot of sample 1 biopsies in collagenase solution

The bile particles were still present in sample 2 after two rounds of PBS wash:
Video snapshot of sample 2 biopsies after thawing and washing

Separating the epithelial layer from the rest of the biopsies is always challenging since it is done 'by eye' where a researcher decides what to call 'a remaining biopsy chunk' and what to keep in the EDTA fraction:
Video snapshot of sample 2 biopsies after 15 min in EDTA

Sample 1 dissociated as expected (photo 3):

- after 25 min in collagenase, biopsy pieces were easily going through p200 tip and even through p10 tip
- there was a decent pellet before filtering
- some tissue was left on the filter (as we usually also have with gut biopsies)
- hard-to-see pellet after filtering

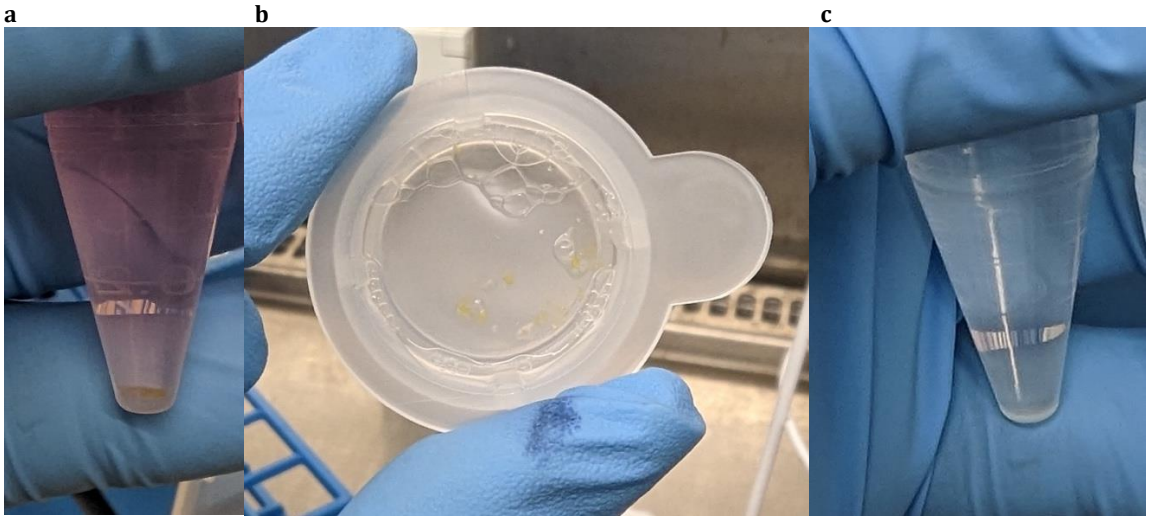


Photo 3. Dissociation of sample 1 (p-0002) – pellet after collagenase step before filtering (a), leftover pieces on the filter (b) and nearly invisible pellet after filtering (c)

Sample 2b (collagenase fraction), photo 4:

- heavily contaminated by bile particles
- some of the particles (cells too?) are very sticky to the plastic tip and impossible to wash out of the tip
- these particles easily pellet and go through the filter, rendering them very hard to get rid of despite the many washing steps

Sample 2a (EDTA fraction):

- heavily contaminated by bile particles
- pellets always contained these particles regardless the washing or filtering

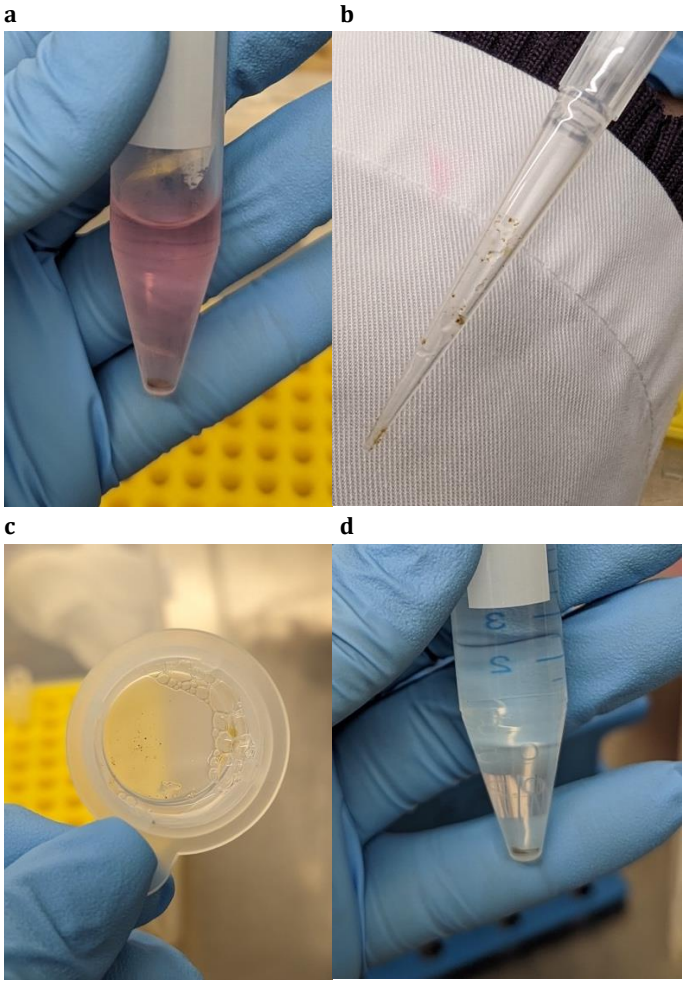


Photo 4. Dissociation of sample 2b (p-0004, collagenase fraction) - pellet after collagenase step before filtering (a), sticky residue in the pipet tip that is impossible to wash out (b), leftover pieces on the filter (c) and pellet after filtering still containing bile particles (d).

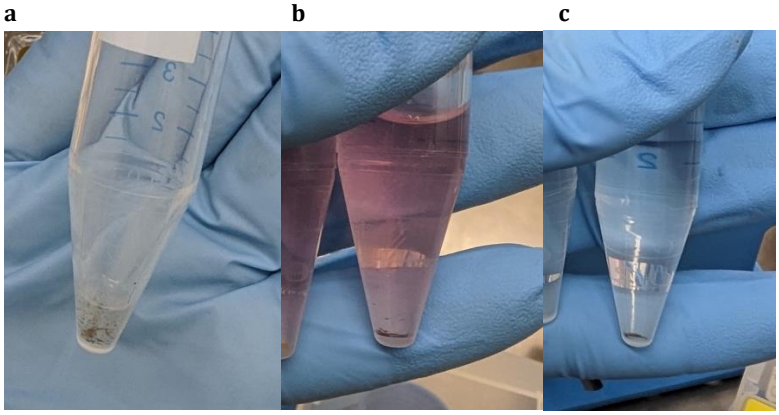


Photo 5. Dissociation of sample 2a (p-0004, EDTA fraction) – pellet after 15 min in EDTA solution, washed in PBS (a), pellet after 1.5 min in TryplE, washed in RPMI/2% FCS (b), and pellet after filtering (c).

Cell counting

After filtering and washing the pellet, the cells were counted manually with trypan blue:

Sample	All cells (average of 2 counts)	Dead cells (average of 2 counts)	Viability %	Total cells (in 50 uL)
Sample 1	8	2	75	8000
Sample 2a	21.5	2.5	88	21.500
Sample 2b	58.5	8	86	58.500

Sample 1 looked clean under the microscope although did not have a high cell count. Both sample 2a and 2b had a lot more cells but also a lot more contamination by bile particles that appeared to be of similar size to cells (Photo 6). That meant that these particles can't be removed by filtering, and if loaded to 10x chip, they can either clog the microfluidics system of the chip or be taken up into GEMs instead of cells, thereby significantly reducing the efficiency of the 10x Genomics protocol.

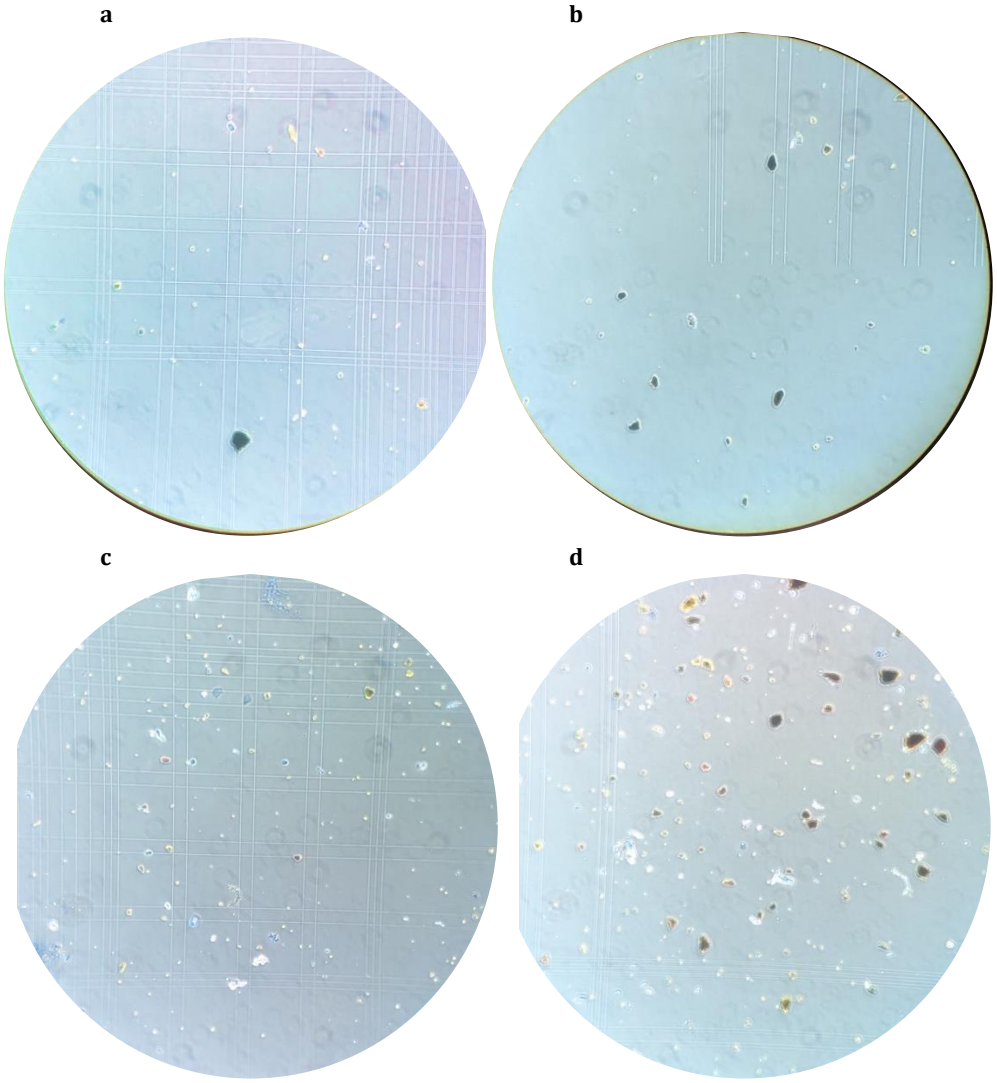


Photo 6. Brightfield microscopy images of the sample 2 cell suspensions: sample 2a, EDTA fraction (a and b), and sample 2b, collagenase fraction (c and d). Single alive cells appear bright white/yellowish round shape, while contaminating bile is either large dark brown or dark yellow irregular shaped particles. By eye, it would seem that EDTA fraction contained larger size cells (i.e. epithelial cells) than collagenase fraction that should be enriched in immune cells.

After counting, cell suspensions were loaded to the 10x chip in the following manner:

- Lane 1 contained max volume (43,2 uL) of cell suspension of sample 1, which translates to ~6900 loaded cells
- Lane 2 contained 2.5:1 ratio of sample 2a (edta): sample 2b (collagenase), which translates to ~9600 sample 2a and ~9900 sample 2b loaded cells

Library construction

The 10x chip loading and the library construction were performed according to the Chromium Next GEM Single Cell 3' Reagent Kits v3.1 (Dual Index), Revision E protocol.

As expected, lane 1 (sample 1) had no issues when loaded onto 10x chip, while lane 2 (sample 2a and 2b) had a severe clogging issue resulting in incomplete GEM formation (photo 7)

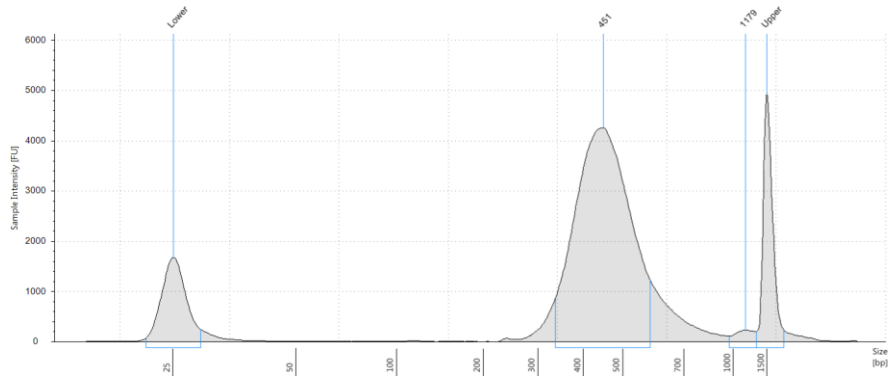
Photo 7. The 10x chip after completed run (a and b), and collected GEMs for further library construction (c). On panel (a), the top row (row 3) are the wells with the resulted GEMs, less volume and less opaque fluid indicate the clog which is clearly visible on panel (c) where the fluid with GEMs has been transferred to the PCR tubes. For lane 2, in row 1

where the cell suspensions are loaded, the precipitated bile particles are clearly visible at the bottom of the well which caused the clog.

Outcome

Only 10x-lane 1 was taken for further library construction. After fragmentation, ligation, indexing and SPRI cleanup, the final product looked like this:

E2: 230511_lane1_cDNAlib

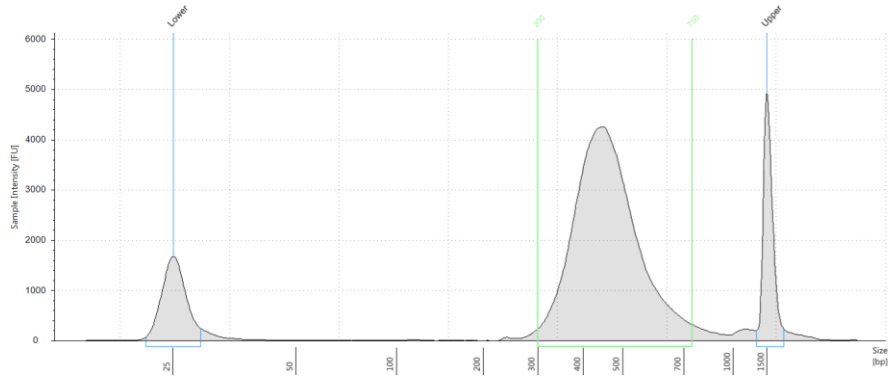


Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
E2	33.5	230511_lane1_cDNAlib		

Peak Table

Size [bp]	Calibrated Conc. [ng/µl]	Assigned Conc. [ng/µl]	Peak Molarity [nmol/l]	%Integrated Area	Peak Comment	Observations
25	5.76	-	354	-		Lower Marker
451	32.9	-	112	98.09		
1179	0.641	-	0.837	1.91		
1500	6.50	6.50	6.67	-		Upper Marker



Region Table

From [bp]	To [bp]	Average Size [bp]	Conc. [ng/µl]	Region Molarity [nmol/l]	%of Total	Region Comment	Color
300	750	468	37.4	127	92.69		

Photo 8: TapeStation D1000 QC report showing a clean cDNA product with majority of fragments falling into 400-500 bp region, main peak at 451 bp, final concentration 37.4 ng/uL

The generated library was sent to BGI sequencing facility on 31-05-2023.
Sequencing length: PE28+10+10+90
Sequencing depth: 55.000 reads/cell
Expected cell recovery (45-65%): 3100 – 4480 cells
Expected data delivery: 01-07-2023