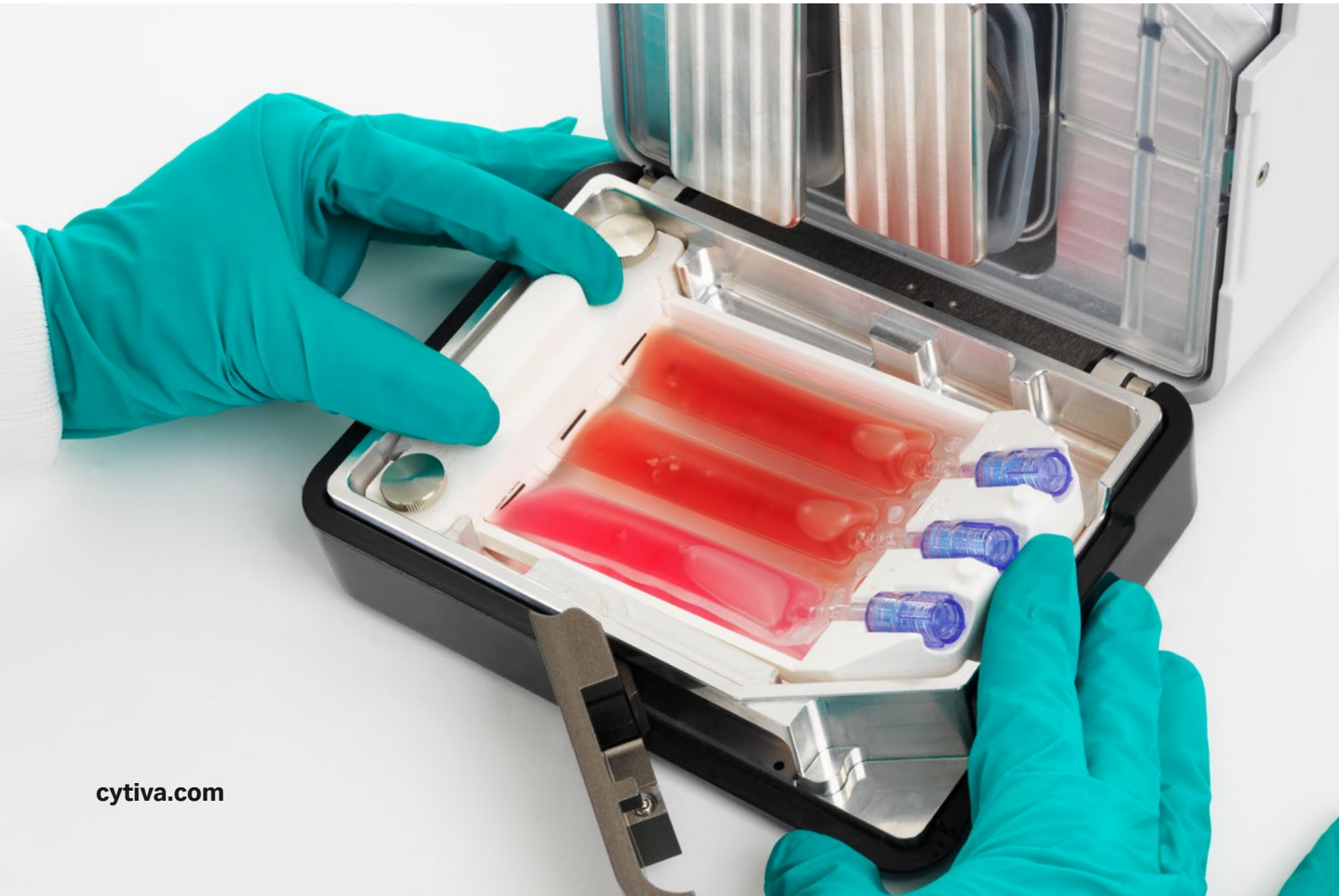
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Case study collection

Tissue dissociation for single-cell sequencing



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Content

Introduction / foreword 3

High-quality single-cell suspensions from heart tissue 4
An investigation to generate high-quality single-cell suspensions from heart tissue shows that VIA Extractor™ tissue disaggregator performs well when comparing yield, cell viability, and single-cell RNA (scRNA) sequencing data.

Quality tissue dissociation for single-nuclei RNASeq preparation 13
An investigation demonstrating that the VIA Extractor™ tissue disaggregator can efficiently dissociate both fresh and snap-frozen tissue into single-nuclei suspensions for downstream sequencing and subsequent analyses.

Comparative study of VIA Extractor™ tissue disaggregator for tissue dissociation for single-cell RNA sequencing analysis 21
An investigation into the ability of the VIA Extractor™ tissue disaggregator to generate suspensions of high-quality single cells from fresh tissue for use in scRNA-seq analysis compared with the gentleMACS dissociator (Miltenyi Biotec).

Introduction / foreword

Historically, sequencing technology enabled only an average analysis of a total cell population. Recent technological advancements have made single-cell sequencing an increasingly powerful tool for understanding biology and cellular function, disease diagnosis, therapy response prediction, and treatment selection. Single-cell sequencing allows tens of thousands of individual cells from a single tissue sample or patient to be analyzed and gives researchers an unprecedented opportunity to understand individual cell populations and their behavior in diseased tissue.

Careful tissue processing is therefore becoming even more important for ensuring that accurate results are achieved from downstream processes. After collection, tissue samples need to be processed and single cells isolated as quickly as possible to make sure cell viability remains high and to minimize changes in the cellular transcriptome. When processing a fresh tissue sample, the disaggregation processes can still result in sample loss via ruptured or attached cells that are then unsuitable for library preparation. Tissue samples can also vary in extracellular matrix (ECM) composition, cell heterogeneity, and rigidity. Consequently, dissociation approaches require extensive optimization and care, as improperly disaggregated samples can reduce cell viability, decrease the efficiency of cell compartmentalization, and block downstream instrumentation.

This collection of case studies highlights ways to standardize the digestion and disaggregation of solid tissue samples to consistently obtain high-quality, viable, single-cell solutions from a range of sample inputs.

High-quality single-cell suspensions from heart tissue

An investigation to generate high-quality single-cell suspensions from heart tissue shows that VIA Extractor™ tissue disaggregator performs well when comparing yield, cell viability, and single-cell RNA (scRNA) sequencing data.

By Rachel Raybould, Development Scientist Single Cell

Introduction

Single-cell omics has rapidly become the method of choice to investigate cellular heterogeneity among cell populations. To prevent bias in any experiment for single-cell analysis, you need to extract, handle, and process the tissue quickly and with care. So, creating a single-cell suspension from tissue can be long and laborious. To save time, you want to choose equipment that speeds up the single-cell workflow while being gentle on the cells and preserving the original cell state as much as possible. The VIA Extractor™ tissue disaggregator* (Cytiva) provides low-impact, fast, and gentle tissue dissociation into single-cell suspensions.

*For research use only. Not for diagnostic use.

In a previous VIA Extractor™ tissue disaggregator case study for mouse liver tissue, we demonstrated that the VIA Extractor™ tissue disaggregator produces cells of a higher yield and less cellular fragility when compared with liver cell suspensions dissociated on the gentleMACS system (Miltenyi Biotec). In this case study, we investigate the ability of the VIA Extractor™ tissue disaggregator to generate suspensions of high-quality single cells from a tougher tissue type: the heart. We compared the performance, in terms of yield, cell viability and single-cell RNA (scRNA) sequencing data, of the VIA Extractor™ tissue disaggregator with the gentleMACS Octo (Miltenyi Biotec).

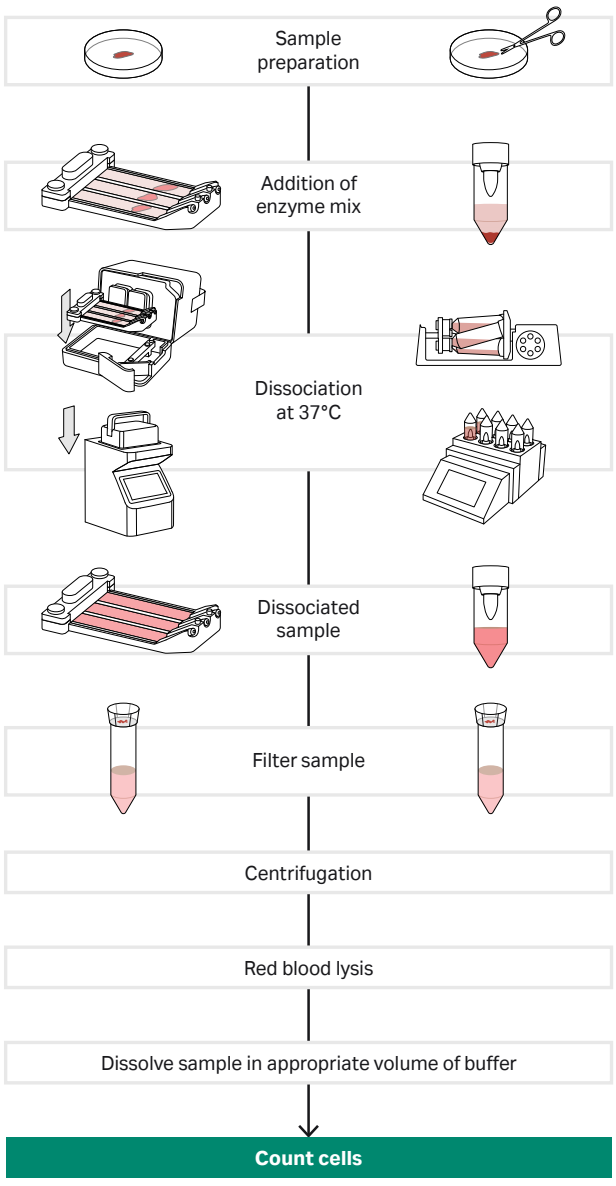
Method

Mouse heart tissue was obtained from 12 female Crl:CD1 (ICR) mice and washed in ice cold phosphate buffer saline (PBS). Forceps were used to pump blood out of each heart and any connective tissue was removed. The mouse hearts were halved into paired samples: half were used for dissociation on VIA Extractor™ tissue disaggregator and half for dissociation on gentleMACS Octo. Half hearts from mice 1, 2, 3, and 4 made up samples V1 and GM1. Half hearts from mice 5, 6, 7, and 8 made up samples V2 and GM2. Half hearts from mice 9, 10, 11, and 12 made up samples V3 and GM3. Equal amounts of tissue and Miltenyi Biotec multi tissue dissociation kit reagents (Miltenyi Biotec) were added to each experimental replicate (Fig 1 and Table 1).

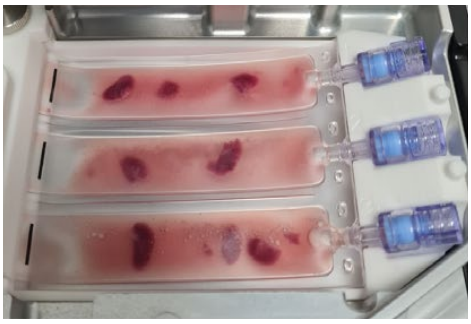
For tissue dissociation on the gentleMACS Octo, Miltenyi Biotec's multi-tissue dissociation kit 2 protocol was followed. For tissue dissociation on the VIA Extractor™ tissue disaggregator, the tissue was dissociated at 37°C for 30 minutes at 200 rpm using Miltenyi Biotec's multi tissue dissociation kit 2 enzyme kit (Table 2).

(A) **VIA Extractor™ tissue disaggregator workflow**

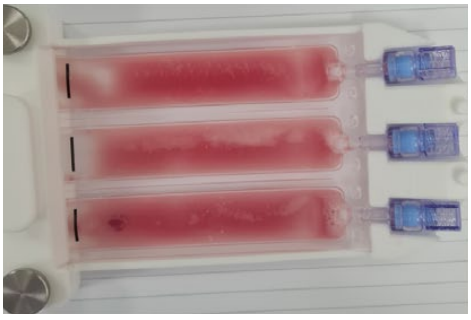
(B) **gentleMACS Octo workflow**



(C) **Heart samples**



Un-dissociated



30 min

(D) **Dissociated samples**

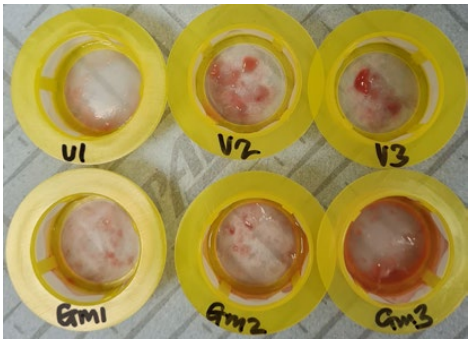


Fig 1. Tissue dissociation workflow for VIA Extractor™ tissue disaggregator and gentleMACS Octo: (A) VIA Extractor™ tissue disaggregator workflow (B) gentleMACS Octo workflow (C) Heart samples were fully dissociated on the VIA Extractor™ tissue disaggregator in 30 minutes. (D) Top view of cell strainer with dissociated samples from the VIA Extractor™ tissue disaggregator (samples V1, V2, and V3) and gentleMACS Octo (samples GM1, GM2, and GM3).

Following dissociation, the sample IDs were re-labelled with the intent to process the samples blind through red blood cell lysis, debris removal, cell capture, and library preparation to minimize bias. All cell suspensions were passed through 100 µm cell strainers and subjected to red blood cell lysis and debris removal using Miltenyi Biotec's red blood cell lysis and debris removal kits. Cells were counted in duplicate using a NucleoCounter® NC-200 (ChemoMetec) and Via2-Cassettes (ChemoMetec). The manufacturer's instructions for the Chromium Next GEM Single Cell 3' dual index kit v3.1 (10X Genomics) were followed with the aim to sequence 1000 cells for each sample. A Chromium Controller (10X Genomics) was used to capture the cells into gel beads in emulsion (GEMs).

Libraries were sequenced on a NextSeq 550 Base (Illumina, Inc.) using NextSeq 550 high output kit v2.5 (Illumina, Inc.). The scRNA matrix data were analyzed using Uniform Manifold Approximation and Projection (UMAP) (1) in Seurat (2). Each sample was analyzed individually, filtered to remove duplicates, include cells with feature RNAs between 200 and 6000, and remove all cells with a mitochondrial gene expression percentage greater than 5%. Once all samples were filtered and clustered, the data from each sample were combined into a single dataset to allow comparison of the scRNA data from the VIA Extractor™ tissue disaggregator and gentleMACS Octo using Seurat (2) and UMAP (1). Cell types representing each cluster were identified using Seurat (2) and marker genes identified by Litviňuková et al. (3). Gene lists from cell clusters with differential gene expression profiles were further analyzed using the gene ontology software package PANTHER (4). All ANOVA tests were performed in JMP Statistical Discovery software by SAS.

Table 1. Tissue weights for each sample dissociated on the VIA Extractor™ tissue disaggregator and gentleMACS Octo. Hearts from mice 1 to 4 were halved and divided equally between samples V1 and GM1. Hearts from mice 5 to 8 were halved and divided equally between samples V2 and GM2. Hearts from mice 9 to 12 were halved and divided equally between samples V3 and GM3.

Mouse	Weight of hearts for VIA Extractor™ tissue disaggregator (g)	Weight of hearts for gentleMACS Octo (g)	Enzyme used	Vol of enzyme (mL)
1, 2, 3, and 4	Sample V1=0.517	Sample GM1=0.511	Multi Tissue Kit 2 (Miltenyi Biotec)	2.5
5, 6, 7, and 8	Sample V2=0.331	Sample GM2=0.389	Multi Tissue Kit 2 (Miltenyi Biotec)	2.5
9, 10, 11, and 12	Sample V3=0.409	Sample GM3=0.426	Multi Tissue Kit 2 (Miltenyi Biotec)	2.5

Table 2. Temperature, speed, and time conditions for dissociation on VIA Extractor™ tissue disaggregator and gentleMACS Octo.

	VIA Extractor™ tissue disaggregator	gentleMACS Octo
Program used	N/A	37C_Multi_G
Program speed	200 rpm	N/A
Program time	30 minutes	56 minutes
Program temperature	37°C	37°C

Results

This study demonstrates that the VIA Extractor™ tissue disaggregator offers a fast and gentle approach to tissue dissociation with improved scRNA sequence data quality but without any impact on cell viability.

The sample preparation time, in terms of washing the tissue and removing blood, for both methods of dissociation was comparable. However, tissue dissociation on the VIA Extractor™ tissue disaggregator was significantly faster compared with the gentleMACS Octo. The heart tissue sample was fully dissociated in 56 minutes using the gentleMACS Octo, but took only 30 minutes to dissociate using the VIA Extractor™ tissue disaggregator (Fig 1B).

The reduction in dissociation time did not impact the yield of cells dissociated from the heart tissue. On the contrary, the cell yield from the VIA Extractor™ tissue disaggregator was more than double that of the gentleMACS Octo. The VIA Extractor™ tissue disaggregator resulted in a higher cell yield (cells per milligram tissue) compared to the gentleMACS Octo (*t* test p = 0.0392, df = 2) (Fig 2).

Both methods of dissociation produced cells that were more than 90% viable after red blood cell lysis and debris removal. The cells were of excellent quality for single-cell sequencing.



Fig 2. Comparison of cell yield between the VIA Extractor™ tissue disaggregator and the gentleMACS Octo (*t* test p = 0.0392, df = 2). The VIA Extractor™ tissue disaggregator results in higher cell yield compared to gentleMACS Octo.

Table 3. The cell count per milligram of tissue is higher in hearts dissociated with the VIA Extractor™ tissue disaggregator compared with the gentleMACS Octo. There is no difference in viability between VIA Extractor™ tissue disaggregator and gentleMACS Octo.

New Sample ID	Dissociation method	Viabilty (%)	Yield (cells/mg tissue)
1A	gentleMACS Octo (GM1)	95	695
2A	VIA Extractor™ tissue disaggregator (V1)	97	3404
3A	VIA Extractor™ tissue disaggregator (V2)	95	5468
4A	gentleMACS Octo (GM2)	94	838
5A	gentleMACS Octo (GM3)	91	822
6A	VIA Extractor™ tissue disaggregator (V3)	96	2445

DRAGEN (Illumina, Inc.) single-cell data analysis indicated that the DRAGEN QC metrics were different between cells dissociated on the VIA Extractor™ tissue disaggregator and gentleMACS Octo. Knee plots from the VIA Extractor™ tissue disaggregator were also observed to be steeper than those of the gentleMACS Octo (Fig 3). Typically, a knee plot from a good quality sample with healthy cell membranes will display a flat plateau followed by a steep drop off. A steep drop off means that there is a clear difference between barcodes that are associated with cells and barcodes that are noncellular. A curve indicates that there is "noise" of noncell associated barcodes that is normally associated with extracellular RNA contamination. The knee plot data support the notion that VIA Extractor™ tissue disaggregator offers a more gentle approach to tissue dissociation. It is possible that the gentleMACS Octo produces cells that are viable but potentially too fragile to survive the GEM capture process on the Chromium Controller. The fragile cells may break and lead to extracellular RNA that reduces the distinction between cell-based barcodes and noncell-based barcodes.

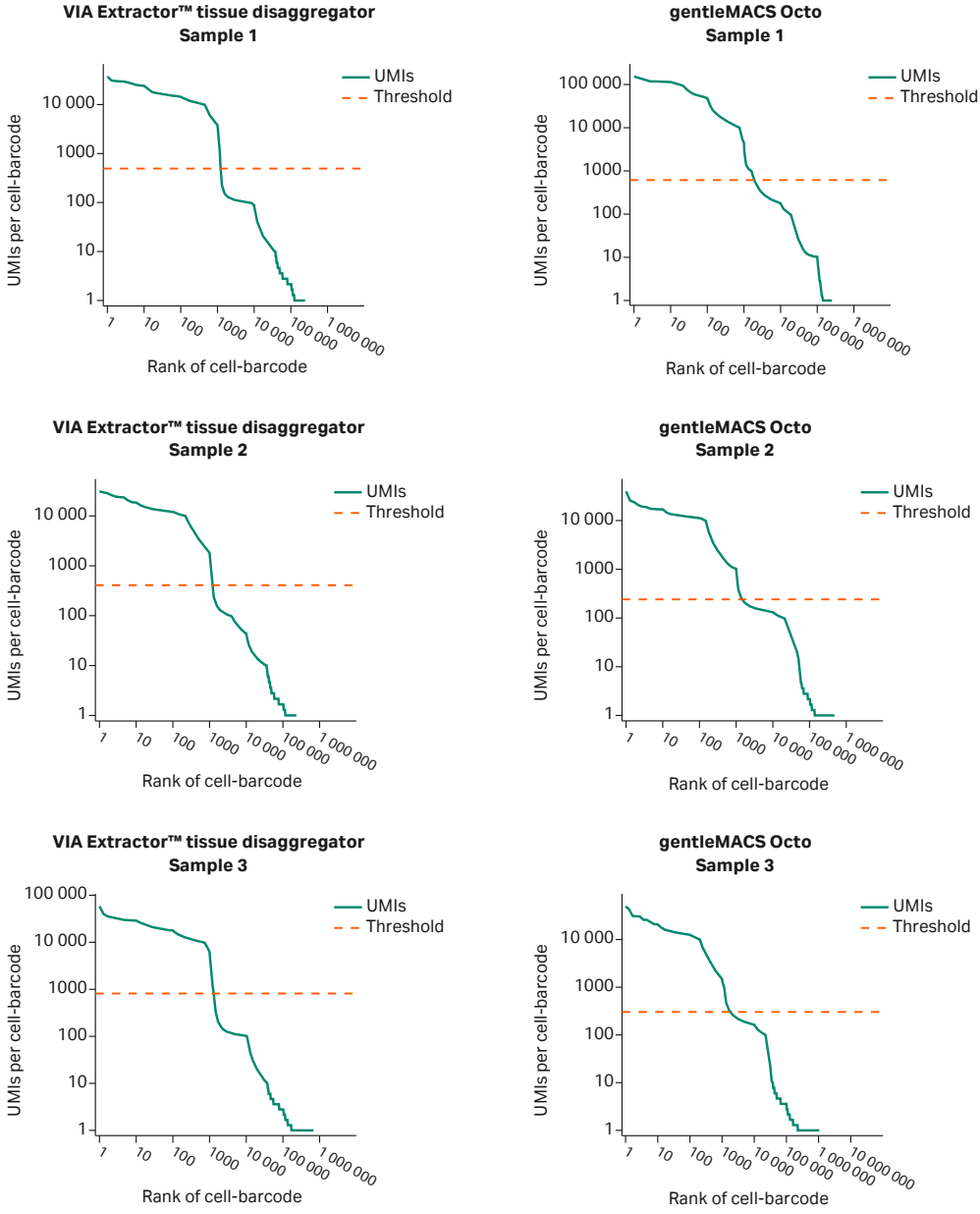


Fig 3. Knee plots for each of the samples post analysis with DRAGEN single-cell RNA application. The samples dissociated on the VIA Extractor™ tissue disaggregator display well-defined knee plots with steep drop offs, whereas samples dissociated on gentleMACS Octo have poorly defined drop offs. A steep drop off is indicative of good separation between the cell-associated barcodes and the barcodes associated with empty partitions. A poorly defined drop off indicates contamination of empty partitions and potentially cell-associated barcodes containing cell free RNA.

The data from the knee plots are supported by other DRAGEN single-cell metrics such as median gene counts per cell and number of cells passing (Fig 4 and 5). The VIA Extractor™ tissue disaggregator had a higher number of cells passing metric thresholds compared with gentleMACS Octo (Fig 4). The median number of gene counts per cell for VIA Extractor™ tissue disaggregator was 1705 ± 122 compared with 617 ± 509 for the gentleMACS Octo. This is statistically significant (*t* test $p = 0.023$, $df = 1$) (Fig 5). The large standard deviation for the gentleMACS Octo samples indicates that the number of gene counts varies considerably between cells, which could be a result of the contaminating barcodes in the cells. These data support the observation made above for the knee plots. In summary, a higher median gene count with a smaller standard deviation and more cells passing are achieved with the VIA Extractor™ tissue disaggregator, which indicates improved quality of cells.

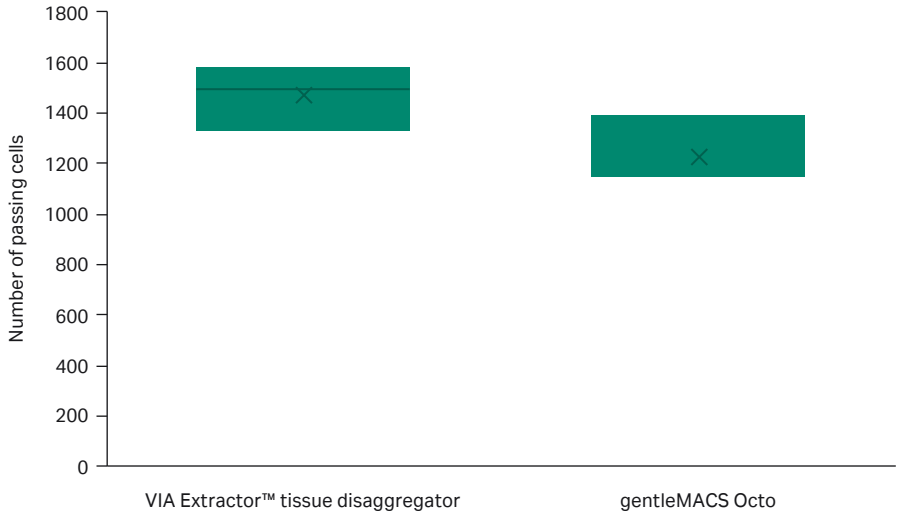


Fig 4. Comparison of the number of cells passing DRAGEN single-cell RNA quality checks. The number of cells passing DRAGEN single-cell RNA quality checks is higher with cells dissociated on VIA Extractor™ tissue disaggregator compared with gentleMACS Octo (*t* test $p = 0.0492$, $df = 4$).

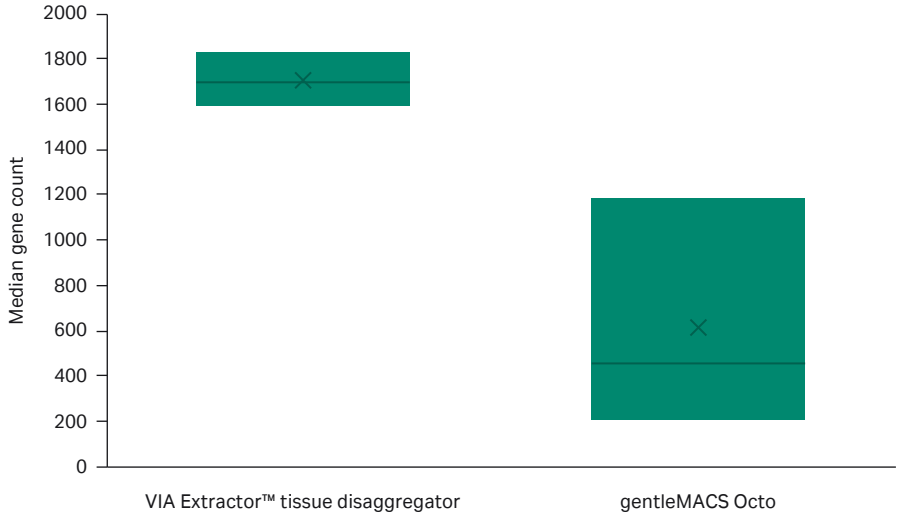


Fig 5. Comparison of the median gene count between VIA Extractor™ tissue disaggregator with the gentleMACS Octo following DRAGEN single-cell RNA quality checks. The number of cells passing DRAGEN single-cell RNA quality checks is higher with cells dissociated on VIA Extractor™ tissue disaggregator compared with gentleMACS Octo (*t* test $p = 0.0295$, $df = 2$).

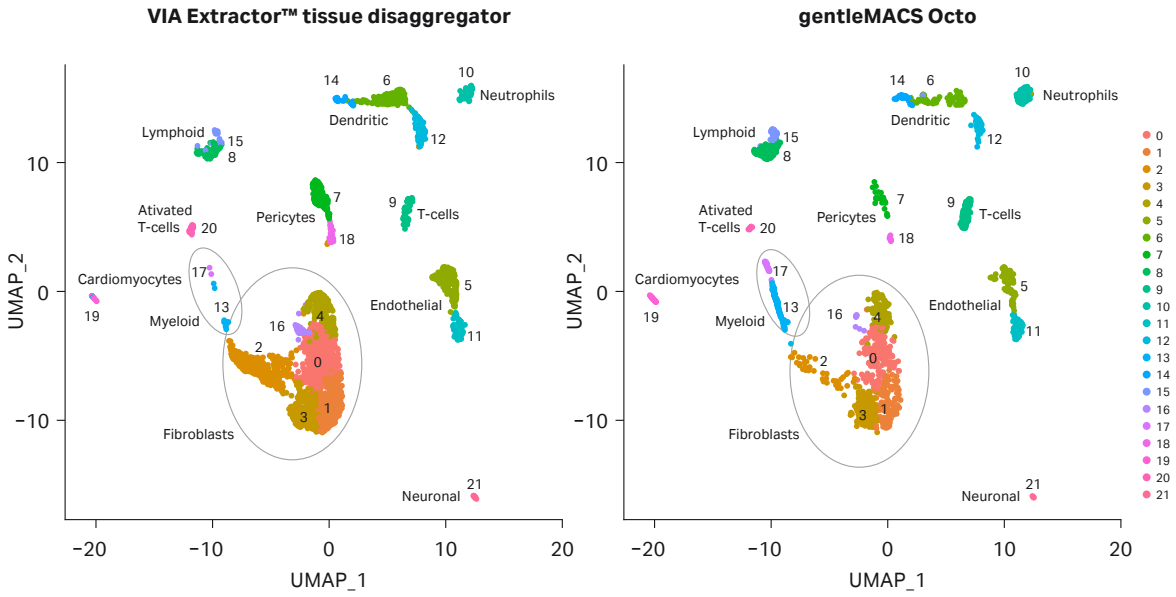


Fig 6. UMAP clustering of cell samples. Following sequencing, 21 clusters were automatically generated for both sample sets by Seurat analysis and cell types assigned.

UMAP analysis uncovered 21 cell clusters and cell types for tissue samples dissociated on both the gentleMACS Octo and VIA Extractor™ tissue disaggregator (Fig 6). There were differences in the proportion of certain cell clusters (Fig 7). At the extremes (cell count proportions that differed by over 80% or under 20%) were cell clusters 2, 7, 18, 13, and 17. Clusters 2, 17, and 18 were overrepresented in samples dissociated by the VIA Extractor™ tissue disaggregator. Clusters 7 and 18 were easily identified as pericytes as they were expressing pericyte gene markers ATP binding cassette subfamily C member 9 (ABCC9), potassium inwardly rectifying channel subfamily J member 8 (KCNJ8), and regulator of G protein signaling 5 (RGS5), as cited by Litviňuková et al. (3). Cluster 2 was identified as expressing fibroblast markers such as decorin (DCN), gelsolin (GSN), and platelet derived growth factor receptor alpha (PDGFRA). Also, cluster 2 had a significantly lower-fold change expression of genes associated with cell stress response (Table 4). The data for cluster 2 supports that heart fibroblasts are less stressed by dissociation on the VIA Extractor™ tissue disaggregator.

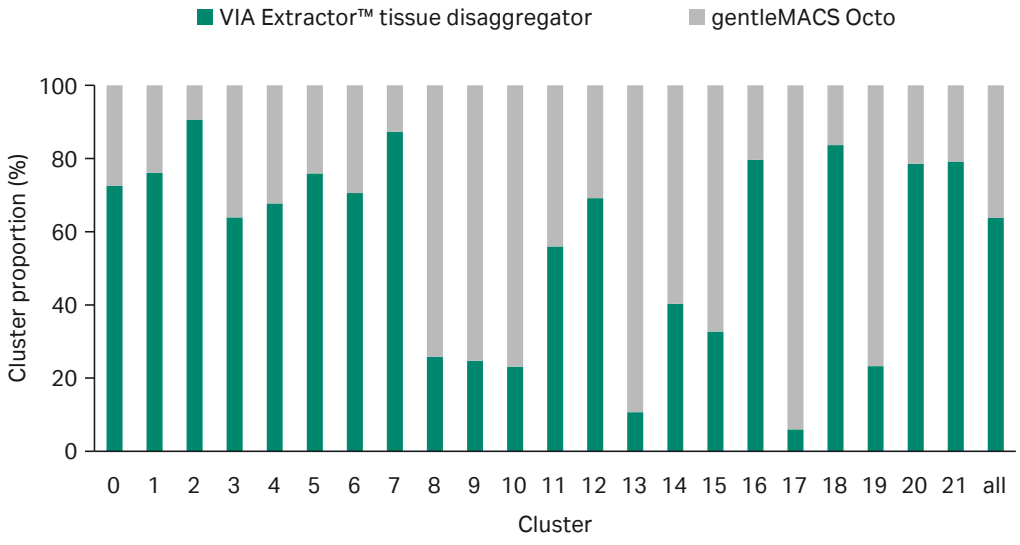


Fig 7. Proportional representation of cells from both sample sets in each of the clusters identified in Figure 6.

Table 4. Gene ontology (GO) biological process analysis using protein analysis through evolutionary relationships (PANTHER) for top 10 genes listed for clusters 2, 13, and 17. Ensembl ID, gene name ID, and log fold change in gene expression (Log-FC exp) for the VIA Extractor™ tissue disaggregator and gentleMACS Octo. Biological process, GO *p*-value and GO false discovery rate (FDR) are shown.

			VIA Extractor™ tissue disaggregator	gentleMACS Octo			
Cluster	Ensembl ID	Gene ID	Log-FC exp	Log-FC exp	GO biological process of all 10 genes	GO p-value	GO FDR
2	ENSMUSG00000052837	Junb	-3.227916943	-3.764941075	negative regulation of transcription from RNA polymerase II promoter in response to stress (GO:0097201)	1.38E-05	9.03E-03
	ENSMUSG00000021250	Fos	-3.770762808	-3.554206217	response to corticosterone (GO:0051412)	2.25E-05	1.31E-02
	ENSMUSG00000052684	Jun	-3.140466151	-3.274124354	integrated stress response signaling (GO:0140467)	2.29E-07	5.12E-04
	ENSMUSG00000020423	Btg2	-3.138476787	-3.097545505	response to muscle stretch (GO:0035994)	4.61E-05	1.90E-02
	ENSMUSG00000092341	Malat1	-2.120789187	-1.760016056	cellular response to calcium ion (GO:0071277)	8.35E-11	1.31E-06
	ENSMUSG00000071076	Jund	-2.102836728	-0.982808053	response to mineralocorticoid (GO:0051385)	9.22E-05	2.63E-02
	ENSMUSG00000091971	Hspa1a	-3.677973078	-2.760131714	cellular response to cadmium ion (GO:0071276)	1.02E-04	2.76E-02
	ENSMUSG00000024190	Dusp1	-2.896103086	-2.236973183	response to progesterone (GO:0032570)	1.07E-04	2.76E-02
	ENSMUSG00000003545	Fosb	-3.01166351	-1.895512893	positive regulation of miRNA transcription (GO:1902895)	1.48E-04	3.26E-02
	ENSMUSG00000086503	Xist	-1.762025276	-0.925347092	skeletal muscle cell differentiation (GO:0035914)	1.60E-04	3.44E-02
13	ENSMUSG00000036887	C1qa	3,488800882	4,760969916	synapse pruning (GO:0098883)	1.93E-08	1.53E-04
	ENSMUSG00000036905	C1qb	3,628318699	4,393600093	cell junction disassembly (GO:0150146)	1,21E-04	3.07E-08
	ENSMUSG00000036896	C1qc	3,345863532	4,070158702	neutrophil activation involved in immune response (GO:0002283)	8.14E-03	1.45E-05
	ENSMUSG00000069516	Lyz2	3,923377675	2,807094198	microglial cell activation (GO:0001774)	6.79E-04	3.02E-07
	ENSMUSG00000058715	Fcer1g	3,257880612	2,359244407	leukocyte activation involved in inflammatory response (GO:0002269)	7.69E-04	4.39E-07
	ENSMUSG00000030579	Tyrobp	3,115315771	2,243030603	glial cell activation (GO:0061900)	8.10E-04	5.65E-07
	ENSMUSG00000024397	Aif1	3,731604102	3,135386677	positive regulation of protein localization to cell surface (GO:2000010)	2.43E-02	6.00E-05
	ENSMUSG00000025150	Cbr2	3,372520543	2,692310302	neutrophil activation (GO:0042119)	2.56E-02	6.48E-05
	ENSMUSG00000069792	Wfdc17	3,752593608	3,369812165	neuroinflammatory response (GO:0150076)	8.73E-04	8.86E-07
	ENSMUSG00000004814	Ccl24	3,047080867	1,309298968	positive regulation of myeloid leukocyte mediated immunity (GO:0002888)	3.09E-02	8.03E-05
17	ENSMUSG00000036353	P2ry12	1,986764773	3,008233495	platelet activation (GO:0030168)	2.91E-02	1.30E-05
	ENSMUSG00000020787	P2rx1	2,066112579	1,733719331	blood coagulation (GO:0007596)	9.19E-05	1.17E-08
	ENSMUSG00000000320	Alox12	3,339230632	3,019615597	coagulation (GO:0050817)	6.47E-05	1.24E-08
	ENSMUSG00000030054	Gp9	2,831003703	3,379404413	hemostasis (GO:0007599)	5.27E-05	1.34E-08
	ENSMUSG00000024511	Rab27b	2,868540327	2,540107035	regulation of body fluid levels (GO:0050878)	1.19E-04	7.59E-09
	ENSMUSG00000032261	Sh3bgrl2	2,827713303	3,069296517	wound healing (GO:0042060)	8.78E-04	2.80E-07
	ENSMUSG00000023993	Treml1	3,389123721	2,330857247	response to wounding (GO:0009611)	2.80E-03	1.07E-06
	ENSMUSG00000046814	Gchfr	1,944583533	2,099188176			
	ENSMUSG00000073414	Mpig6b	2,065347999	1,819772799			
	ENSMUSG00000034664	Itga2b	2,751894914	3,408245231			

Clusters 13 and 17 express myeloid genes such as transmembrane immune signaling adaptor (TYROBP) and triggering receptor expressed on myeloid cells like 1 (TREML1) and were over represented in samples dissociated on the gentleMACS Octo. Clusters 13 and 17 are characterized by over expressing genes that encode proteins involved in the inflammatory response and wound healing, respectively (Table 4). CQ1 is involved in complement activation, response to infection, and removal of apoptotic cells. The gene list for cluster 17 indicates that there is a high level of expression of genes encoding proteins involved in wound healing. The data for cluster 13 and 17 can be interpreted in one of two ways. The first interpretation supports the concept that the VIA Extractor™ tissue disaggregator provides a method of gentle dissociation with less damage to cells, which results in fewer cells expressing genes involved in macrophage activation and response to tissue damage. The second interpretation may indicate that the gentleMACS Octo allows detection of the myeloid cells involved in complement activation and tissue repair.

Conclusion

Semiautomated tissue disaggregation is an effective way of easing the burden of tissue dissociation, which is the first step in a long single-cell sequencing workflow. To reduce biases that may be introduced by sample preparation workflows, it is important to maintain the cell state as near to its original tissue state as possible. To do this, tissue dissociation requires a fast process that will introduce the least amount of stress possible to the cells. We have previously demonstrated with mouse liver tissue that the VIA Extractor™ tissue disaggregator produces cells of a higher yield and less cellular fragility when compared with liver cell suspensions dissociated on the gentleMACS system (Miltenyi Biotec). In this current investigation, we performed tissue dissociation to compare the VIA Extractor™ tissue disaggregator to gentleMACS Octo using three paired samples from mouse heart tissue. Heart tissue is known for its tough and difficult to dissociate nature. We have confirmed that for heart tissue, the VIA Extractor™ tissue disaggregator provides a faster solution with increased yield of cells compared with the gentleMACS Octo. Furthermore, our data demonstrates that the gentle approach of the VIA Extractor™ tissue disaggregator improves the quality of the single-cell RNA sequencing data, reduces the number of cells with fragile cell membranes, and improves cell capture.

This data is based on three independent experiments with the equal number of replicates in each experiment. All samples tested were treated equally (with the number of replicates being the same for all products tested in the comparison) and according to manufacturers' protocol and recommendations. Data was collected at Cytiva, Maynard Centre, Cardiff, UK (R&D Laboratory) during April to August 2022 and is held at this location.

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Quality tissue dissociation for single-nuclei RNASeq preparation

An investigation demonstrating that the VIA Extractor™ tissue disaggregator can efficiently dissociate both fresh and snap-frozen tissue into single-nuclei suspensions for downstream sequencing and subsequent analyses.

By Ben Tivey, R&D Scientist

Introduction

Single-cell RNA sequencing (scRNA-seq) allows researchers to delve deeper into the heterogeneity among cellular populations. For example, clinicians can explore the intratumor genomic heterogeneity of rare cancer biopsies. Single-nuclei RNA sequencing (snRNA-seq) is a technique that uses isolated nuclei, rather than whole single cells, to ascertain the genomic landscape of cellular populations within a given tissue sample. Nuclei are often preferred as a starting material for genomic applications because they can be extracted and processed post-cryopreservation. Whole cells are prone to thawing-induced cell death. Previous comparative studies between snRNA-seq and scRNA-seq concluded that, in adult murine kidney, snRNA-seq provides reduced dissociation bias, abolition of dissociation-induced transcriptional stress responses, and even the ability to process inflamed fibrotic tissue (1).

We previously showed that the VIA Extractor™ tissue disaggregator* (Cytiva) can process fresh tissue samples into single-cell suspensions of high yield and viability. Here, we investigate the ability of the VIA Extractor™ tissue disaggregator to fully dissociate both fresh and snap-frozen tissue into single-nuclei suspensions for downstream sequencing and subsequent analyses.

*For research use only. Not for diagnostic use.

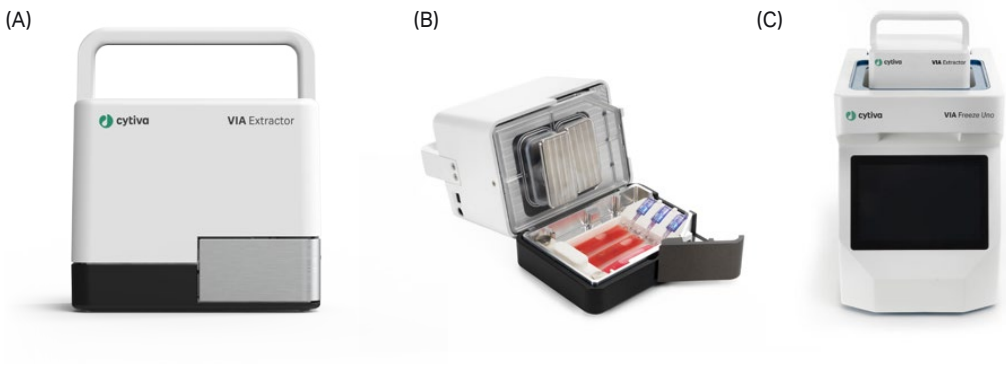


Fig 1. (A) The VIA Extractor™ tissue disaggregator provides fast, minimal-impact tissue dissociation into single-nuclei suspensions, (B) The Omics pouch placed into the VIA Extractor™ tissue disaggregator and held in place with the Omics clamp. (C) The VIA Extractor™ tissue disaggregator placed into the top of the VIA Freeze™ Uno controlled-rate freezer.

Methods

Murine kidney and heart tissues were collected from three freshly dissected Crl:CD1 (ICR) female mice (Charles River). Three kidney pairs and six hearts were carefully separated in half. The heart halves were then divided into paired samples to further reduce sample variability. One half of each tissue type was snap-frozen in liquid nitrogen and placed into -80°C storage. The other halves were washed in ice-cold phosphate-buffered saline (PBS) and weighed (Fig 2).

For nuclei extraction, a modified version of the methods set out by Fish et al. (2) was applied for use with the VIA Extractor™ tissue disaggregator.

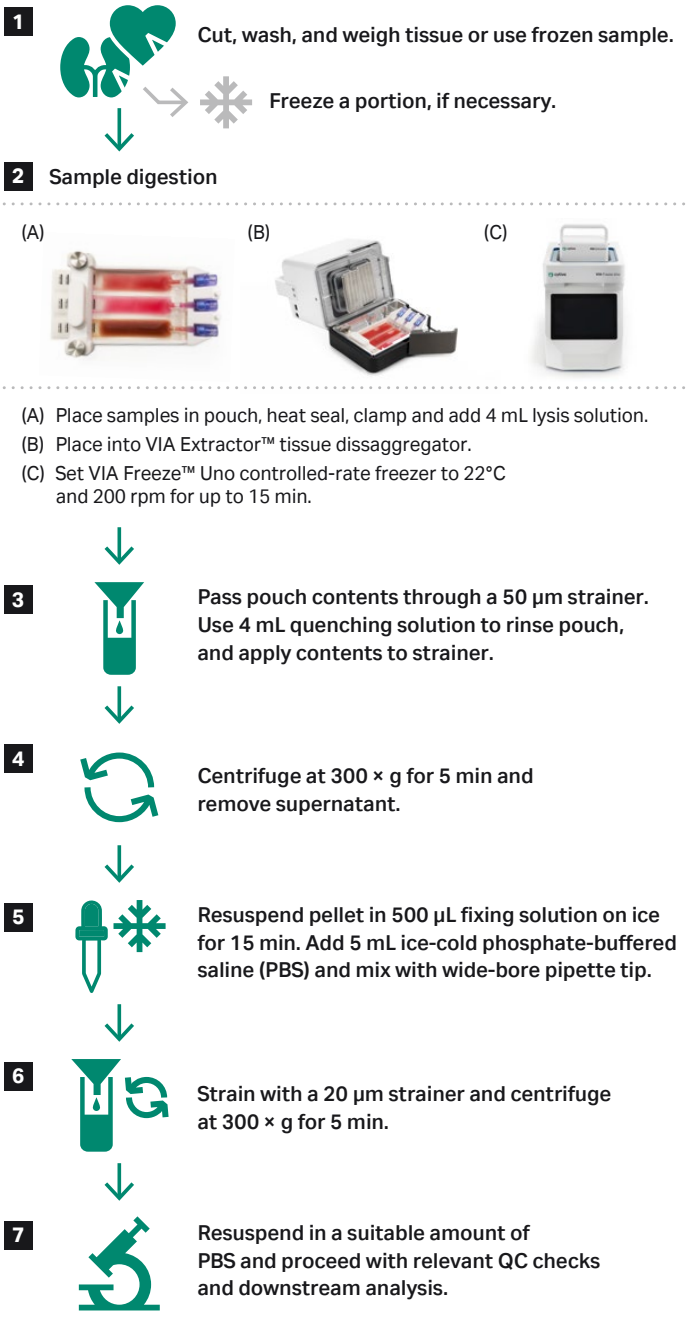


Fig 2. Nuclei suspension preparation workflow using fresh or frozen tissue and the VIA Extractor™ tissue disaggregator in conjunction with the VIA Freeze™ Uno controlled-rate freezer.

For the fresh kidney and heart samples, the tissue was placed into the Omics pouch (Cytiva) using the Omics applicator (Cytiva), heat sealed, and placed into the Omics clamp (Cytiva). When repeated on the frozen batches (which were stored at -80°C for one month), each sample was removed from storage, weighed, and 1 mL of tissue lysis solution was added directly onto the tissue, which allowed the placement of the sample inside of the Omics pouch.

Table 1. Average murine tissue weights per condition, completed in triplicate.

Tissue	Condition	Weight (mg)
Kidney*	Fresh	235
Kidney	Frozen	289
Heart	Fresh	252
Heart	Frozen	188

*Two fresh kidney tissue repeats kept in MACS Tissue Storage Solution (Miltenyi Biotec) at 4°C for 24 hours.

Using a luer lock syringe, 4 mL of tissue lysis solution was added to each section of the Omics pouch and was secured by the Omics Clamp. Samples were processed at 200 rpm and 22°C for 15 minutes.

The contents of each Omics pouch portions were removed from the ports using a 5 mL luer lock syringe and subsequently filtered through a 50 µm cell strainer. Once centrifuged, the supernatant was removed. Then the pellet was resuspended in fixing solution and incubated on ice. A volume of ice-cold PBS was added to the suspension, mixed, passed through a 20 µm cell strainer, and centrifuged under the same conditions. Nuclei were resuspended in ice-cold PBS and counted on NucleoCounter® NC-200 (ChemoMetec A/S) using Via2-Cassette (ChemoMetec A/S) and viewed using brightfield microscopy paired with a hemocytometer. A statistical analysis was completed using JMP 15.2 (SAS Institute).

Results

In the data shown below, we demonstrate that the VIA Extractor™ tissue disaggregator has adaptable capabilities not limited to live, single-cell dissociation but also single-nuclei isolation.

Fresh and frozen samples were dissociated successfully after 15 minutes using the VIA Extractor™ tissue disaggregator at 200 rpm and 22°C (Fig 3).

Table 2. Average nuclei aggregation per tissue type and condition as a percentage with standard error values.

Tissue	Condition	Aggregation (% ± SE)
Kidney	Fresh	2.3 ± 0.7
Kidney	Frozen	2.0 ± 1.0
Heart	Fresh	1.7 ± 0.9
Heart	Frozen	4.3 ± 2.4

To alleviate downstream issues while performing snRNA-seq, keeping aggregation to a minimum is key. Following dissociation of nuclei using the VIA Extractor™ tissue disaggregator, we observed minimal variability among the percentage aggregation between fresh and frozen kidney and heart samples, with all mean averages returning < 5% aggregates in each sample (Table 2; *t* test; Kidney $p = 0.80$; Heart $p = 0.39$).

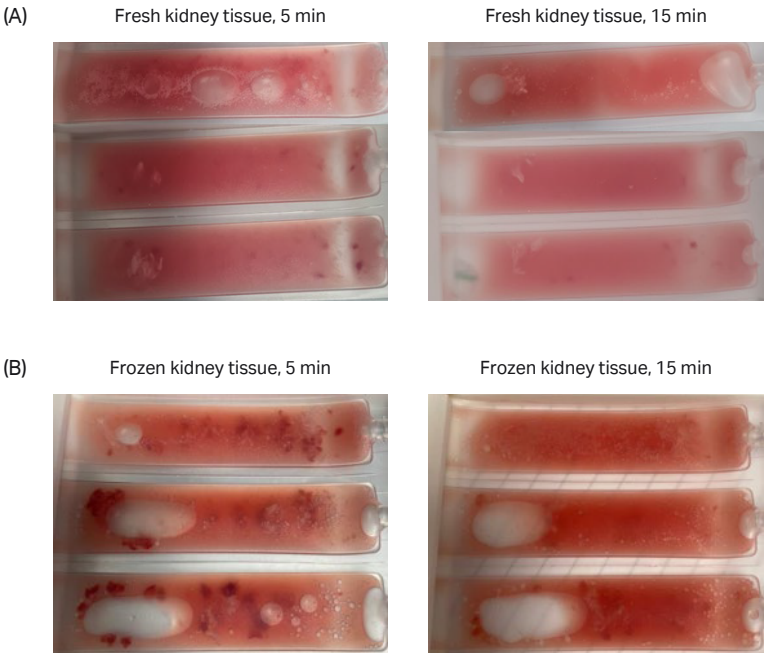


Fig 3. A comparison between fresh and frozen kidney tissue suspension in the Omics pouch 5 minutes into dissociation versus 15 minutes into dissociation. Both fresh and frozen kidney samples were completely dissociated after 15 minutes using the VIA Extractor™ tissue disaggregator.

In conjunction with the automatically calculated numerical aggregation estimate, physical proof of minimal nuclei aggregates is shown in the acridine orange (AO) and 4',6-diamidino-2-phenylindole (DAPI) merged staining (Figs 4 and 5). Because AO and DAPI both have a high affinity for DNA, the images show specific nuclei and their surrounding neighbors in sharp contrast. These data suggest that the VIA Extractor™ tissue disaggregator can reliably prepare nuclei suspensions with a low percentage of aggregates from both fresh and frozen kidney and heart tissue.

Using brightfield microscopy paired with a hemocytometer, we were able to assess the integrity of the nuclear membranes present in each sample. Each brightfield image suggests that the nuclear membranes remain intact (Figs 4 and 5). To further reinforce our observation, in the heart samples specifically, you can easily spot the characteristic elliptical shape of a cardiomyocyte's nucleus (Fig 5). The VIA Extractor™ tissue disaggregator can process tissue and isolate single nuclei without causing damage to the nuclear membrane in kidney and heart tissues.

The scatter plots show the nuclei size distribution and staining intensity of AO and DAPI. Minimal small, dim particles are counted (bottom left of the scatter plots), which suggest that limited amounts of debris are present in both tissue types, regardless of whether the sample is fresh or frozen. The analogous nature of the heatmap-like plotted points within each gate across Figures 4 and 5 further confirm the presence of nuclei post-processing.

Each histogram (Figs 4 and 5) has a regular peak around a cell/nuclei diameter of approximately 9 to 10 µm, which is consistent with the fact that a typical mammalian nucleus is approximately 5 to10 µm (4). The small peak to the right of the major peak in the histograms present in Figure 4 likely represents nuclear doublets, which are found at a much lower percentage of the total material present in the sample.

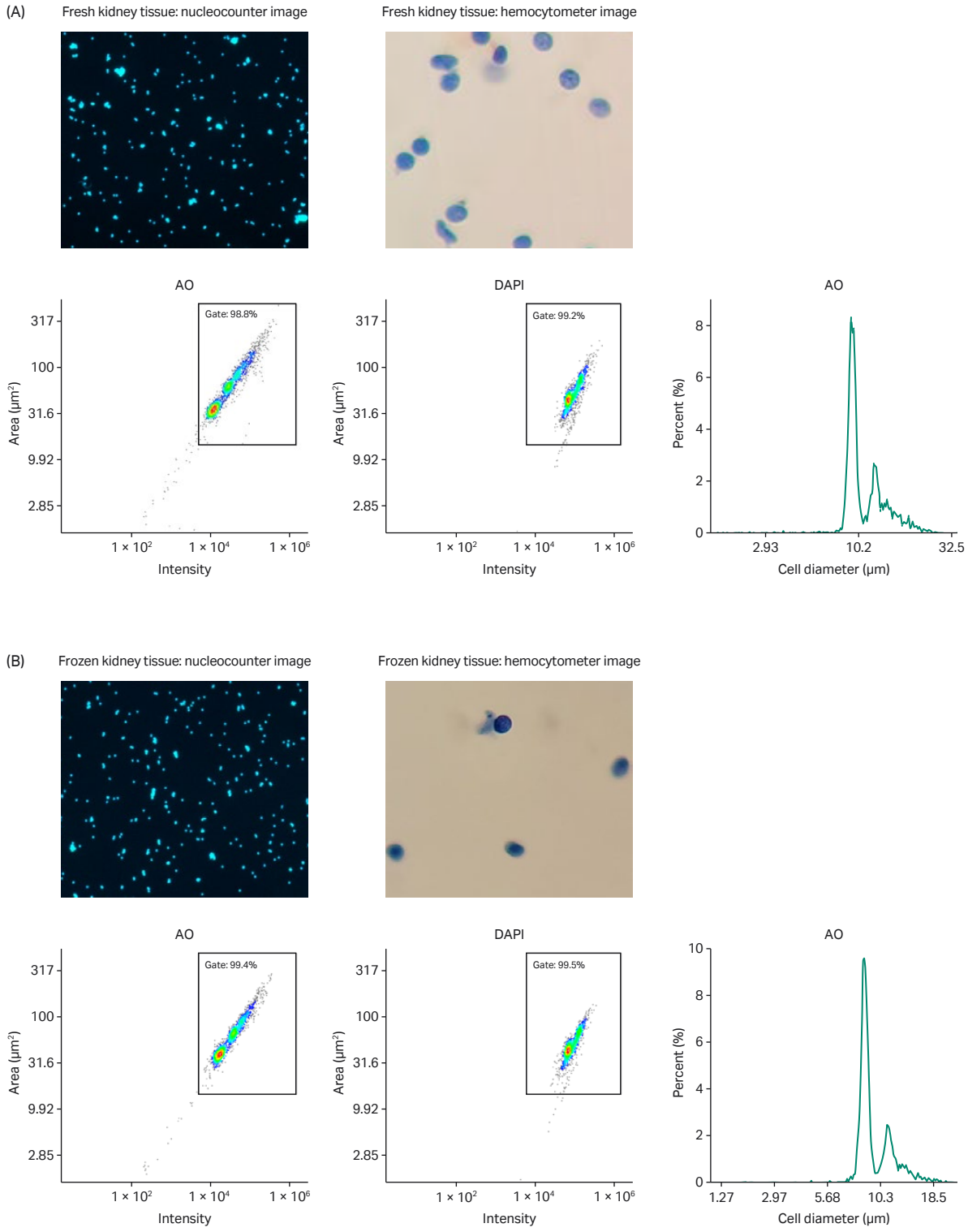


Fig 4. Analysis of single nuclei suspension from (A) fresh and (B) frozen kidney tissue. AO and DAPI merged staining using a NucleoCounter® NC-200 and Via2-Cassette; nuclei-integrity check using brightfield microscopy paired with a hemocytometer; AO intensity versus area scatter plot with automatic gate; DAPI intensity versus area scatter plot with automatic gate; histogram representing estimated cell diameter versus percentage present in sample (cell diameter substituted for nuclei diameter as default).

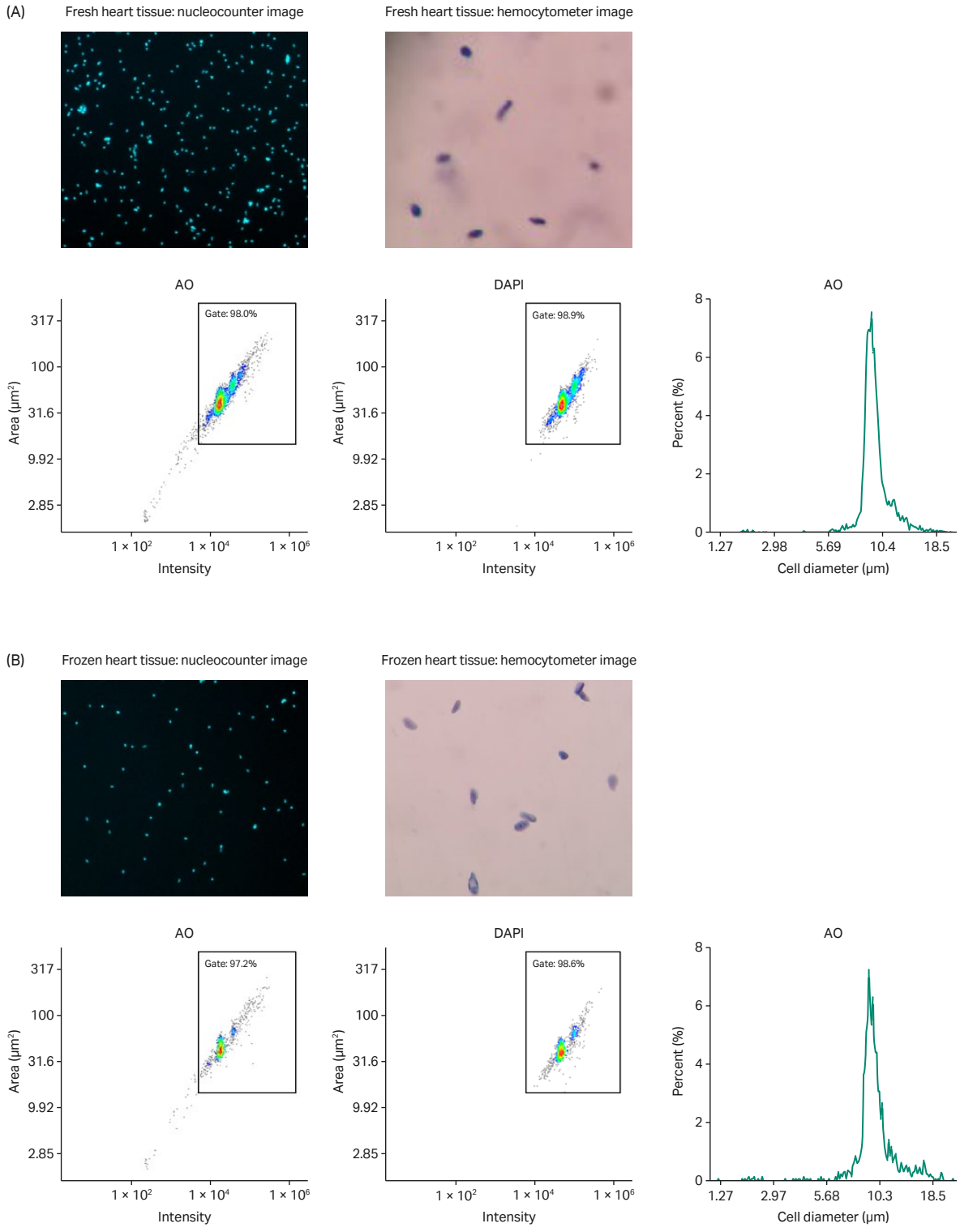


Fig 5. Analysis of single nuclei suspension from (A) fresh and (B) frozen heart tissue. AO and DAPI merged staining using a NucleoCounter® NC-200 and Via2-Cassette; nuclei-integrity check using brightfield microscopy paired with a hemocytometer; AO intensity versus area scatter plot with automatic gate; DAPI intensity versus area scatter plot with automatic gate; histogram representing estimated cell diameter versus percentage present in sample (cell diameter substituted for nuclei diameter as default).

The typical nuclei recovery yield using the Chromium Single Cell Nuclei Isolation Kit (10X Genomics) from healthy frozen tissue ranges from approximately 5 to15 thousand nuclei/mg (5). Our results show proportional consistency between each tissue type, with no significant difference between fresh and frozen samples ($p > 0.05$). The fresh murine kidney samples yielded an average of 52 667 ± 18 559 nuclei/mg, while the yield for the frozen kidney tissue was 49 000 ± 8020 nuclei/mg (Fig 6; *t* test $p = 0.86$). For fresh murine heart tissue samples, the average yield was 7414 ± 762 nuclei/mg; whereas for the frozen heart samples, the yield was 8316 ± 1926 nuclei/mg (Fig 7; *t* test $p = 0.89$).

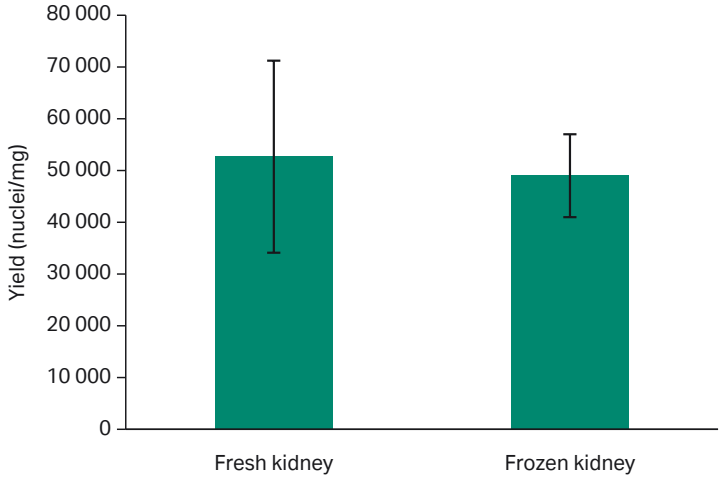


Fig 6. Average nuclei yield per milligram comparison for both fresh and frozen kidney tissue with standard error bars. Fresh: 52 667 ± 18 559 nuclei/mg; frozen 49 000 ± 8020 nuclei/mg (*t* test $p = 0.86$).

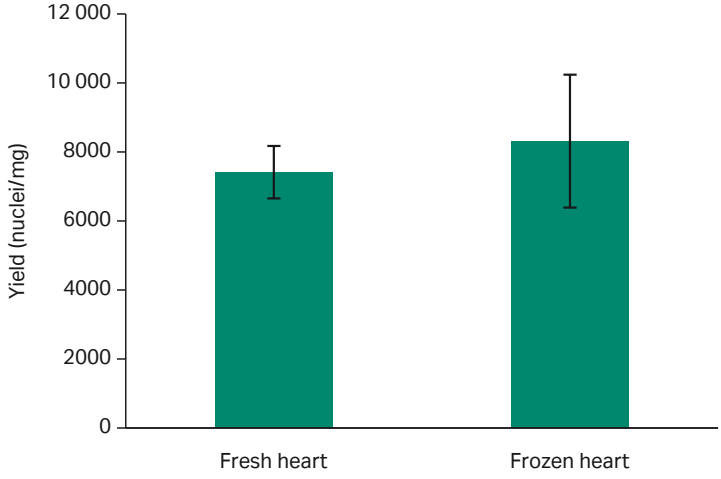


Fig 7. Average nuclei yield per milligram comparison for both fresh and frozen heart tissue with standard error bars. Fresh: 7414 ± 762 nuclei/mg; Frozen 8316 ± 1926 nuclei/mg (*t* test $p = 0.89$).

Conclusion

We previously demonstrated that the VIA Extractor™ tissue disaggregator can produce single-cell suspensions of high yield and viability with low cellular fragility.
Here, we show that the VIA Extractor™ tissue disaggregator can process solid kidney and heart tissue samples into single-nuclei suspensions. Whether the tissue sample is fresh or frozen, the VIA Extractor™ tissue disaggregator can produce consistent and homogenous single-nuclei suspensions with a high yield, intact nuclear membrane, and a low proportion of aggregates. The suspensions could be used further downstream for snRNA-seq and subsequent analyses to ascertain the transcriptional landscape of rare cell populations.

This data is based on three independent experiments with the equal number of replicates in each experiment. All samples tested were treated equally (with the number of replicates being the same for all products tested in the comparison) and according to manufacturers' protocol and recommendations. Data was collected at Cytiva, Maynard Centre, Cardiff, UK (R&D Laboratory) during April to August 2022 and is held at this location.

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Comparative study of VIA Extractor™ tissue disaggregator for tissue dissociation for single-cell RNA sequencing analysis

An investigation into the ability of the VIA Extractor™ tissue disaggregator to generate suspensions of high-quality single cells from fresh tissue for use in scRNA-seq analysis compared with the gentleMACS dissociator (Miltenyi Biotec).

By Devina Divekar, Post Doctoral Genomics Research Fellow, Cytiva and
Rodrigo Grandy-Morgan, Senior Development Scientist - Genomics and Cellular Research, Cytiva

Introduction

Single-cell sequencing is a powerful tool in the study of cellular heterogeneity among individual cells. Advances in single-cell RNA sequencing (scRNA-seq), in particular, have enabled scientists to gain unprecedented insights into transcription profiles at the single-cell level, allowing identification and study of rare cell populations in tissues of interest. Due to the high sensitivity of single-cell analyses, such as scRNA-seq, detailed attention must be put into experimental setup and execution. Careful handling and processing of human or animal tissue sample is critical to minimize any process-induced effects that may skew results. Here, we investigate the ability of the VIA Extractor™ tissue disaggregator* (Cytiva) (Fig1) to generate suspensions of high-quality single cells from fresh tissue for use in scRNA-seq analysis, and compare its performance to the gentleMACS dissociator (Miltenyi Biotec).

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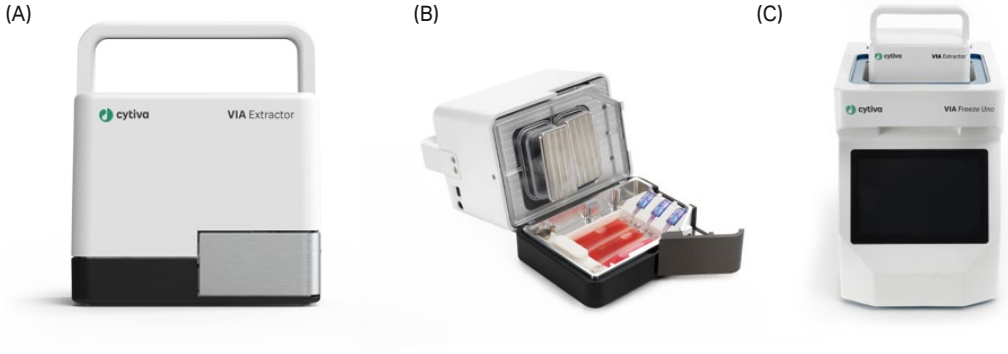
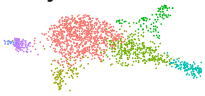


Fig 1. The VIA Extractor™ tissue disaggregator provides fast, low impact tissue dissociation into single-cell suspensions, (B) The Omics pouch placed into the VIA Extractor™ tissue disaggregator and held in place with the Omics clamp. (C) The VIA Extractor™ tissue disaggregator placed into the top of the VIA Freeze™ Uno controlled-rate freezer.

Tissue processing is extremely important in achieving the best results for any downstream single-cell application. In order to obtain a high yield of healthy viable cells, it is important to isolate the tissue of interest while it is as fresh as possible and to process it immediately after collection. During processing, cells are continually responding to changes in their environment. Those responses can carry over into transcription profiles expressed by the cells, so minimizing the impact of processing prior to library preparation is key to ensuring that the best quality data is obtained downstream. A fast, yet gentle tissue dissociation method that is reproducible will help avoid any batch effect or variation due to handling. Table 1 describes the variables and considerations to keep in mind while planning any single-cell sequencing experiment.

Table 1. Overview of stepwise approach to designing single-cell analysis workflow. Adapted from: https://www.frontiersin.org/files/Articles/391125/fcell-06-00108-HTML/image_m/fcell-06-00108-g001.jpg

	Steps	Key variables	Key considerations
1	**Tissue acquisition** 	• Primary human • Model organism	• Biological variation • Sampling variation • Handling variation • Duration of transportation
2	**Tissue disaggregation** 	• Mechanical dissociation • Enzymatic digestion • Automated dissociation • Microfluids device	• Experimental reproducibility • Shortest duration • Maximum viability • Highest quality • Representation of cell types • High yield
3	**Single cell partitioning/ library prep** 	• Droplet based • Tube based after FACS • Microwell based • Microfluids enabled	• Cell throughput and handling time • Gene coverage and cell type detection • Whole transcript versus 3'end counting • Imaging capability for doubtlet detection
4	**Library sequencing** AACTTAATAAACAATTCTGACCTA CAAATTTGTTTATCGTCTAAAAAA ATAAATTAATAAACAATTCTGACC ACCAAATTTGTTTATCGTCTAAAA ATAATAAACAATTCTGACCTACCA	• illumina NGS • Compatible with cDNA library	• 3'end counting: low depth • Whole transcript • Alternative splicing • Iterative optimization for biological system
5	**Computational analysis** 	• Separation of batch and condition • Technical vs biological variation	• Batch correction • Differential expression analysis • Call type identification • Dimensionality reduction

Method

Murine liver tissue was collected from three freshly dissected 129Sv females. After careful isolation, the tissue was weighed and washed with culture media, and liver lobes from each mouse were carefully and equally divided between the VIA Extractor™ tissue disaggregator and gentleMACS to minimize the sample variability (Table 2). Tissue disaggregation protocols were performed according to manufacturer's instructions (Table 3).

All mice chosen were littermates to minimize variability between samples.

Table 2. Tissue weights and the volume of enzyme mix used per sample in both gentleMACS and VIA Extractor™ tissue disaggregator.

Mouse	Liver for gentleMACS	Liver for VIA Extractor™ tissue disaggregator	Enzyme volume
1	400 mg	396 mg	4 mL gentleMACS enzymes
2	399.2 mg	398 mg	4 mL gentleMACS enzymes
3	366.6 mg	369 mg	4 mL gentleMACS enzymes

Table 3. Liver tissue was dissociated using the manufactures guidelines defining the parameters such as speed, time and temperature.

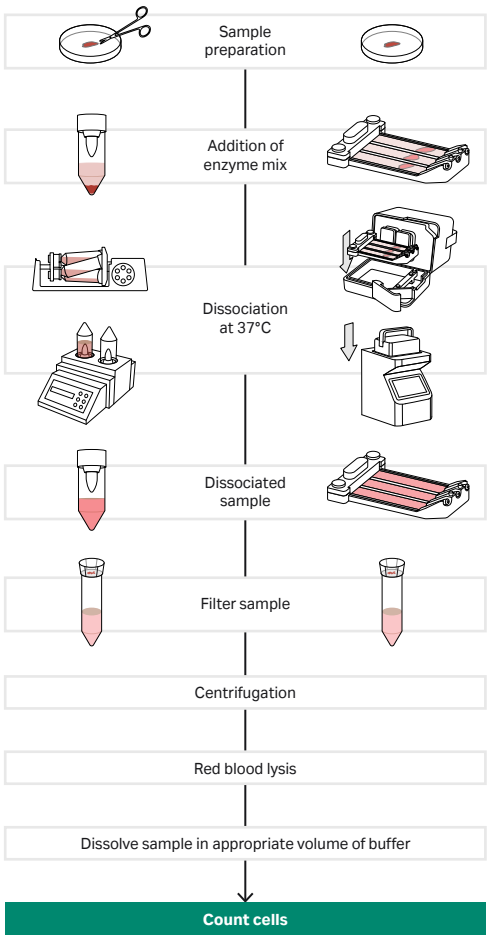
Mouse liver	gentleMACS	VIA Extractor™ tissue disaggregator
Processing speed (RPM)	N/A	200
Time to complete digestion (min)	~37 s processing + 30 min incubation + ~37 s processing	10
Processing temperature	37°C	37°C

For the gentleMACS dissociator, liver tissue was washed with prewarmed Dulbecco's Modified Eagle Medium (DMEM) before adding it to each C tube containing the enzyme cocktail from the Liver Dissociation Kit (Miltenyi Biotec). The C tube was placed on the gentleMACS dissociator and the defined program for liver run for 37 seconds, and then the sample was incubated at 37°C for 30 mins on the MACSmix Tube Rotor (Miltenyi Biotec). Following incubation, each C tube was again placed on the gentleMACS dissociator and the second defined program for liver was run to generate the cell suspension. Following filtration of the samples, tissue debris was clearly visible on the cell strainers indicating that the tissue had not completely disaggregated (Fig 2C iv).

For the VIA Extractor™ tissue disaggregator, liver tissue was washed with prewarmed DMEM before insertion into the Omics pouch. After sealing, the same enzyme cocktail as used for the gentleMACS protocol was added to the pouch via the syringe port. The Omics pouch was then placed into the VIA Extractor™ tissue disaggregator and digestion was carried out for 10 minutes at 37°C. The VIA Extractor™ tissue disaggregator completely dissociated the liver tissue in 10 minutes, with no debris evident on the cell strainers following filtration of the samples (Fig 2D ii and 2D iii).

Following dissociation, all samples from both methods were centrifuged at 300 × g for 10 minutes at 4°C. Supernatants were carefully discarded, and red blood cells removed using the RBC Lysis Buffer (Miltenyi Biotec) according to the manufacturer's protocol. Finally, each sample was carefully diluted to a working concentration of 800 cells/µL. The samples were then immediately processed by the Earlham Institute, Norwich, UK. Following quality evaluation, single cells produced from each system were processed using the Chromium controller (10x Genomics) and libraries were sequenced using the NovaSeq 5000 platform (Illumina).

(A) **gentleMACS workflow** (B) **VIA Extractor™ tissue disaggregator workflow**



(C) **gentleMACS assembly for tissue disaggregation**

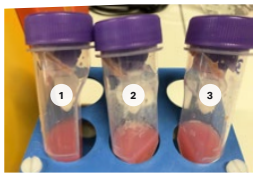
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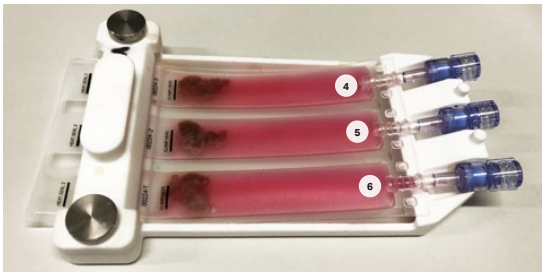


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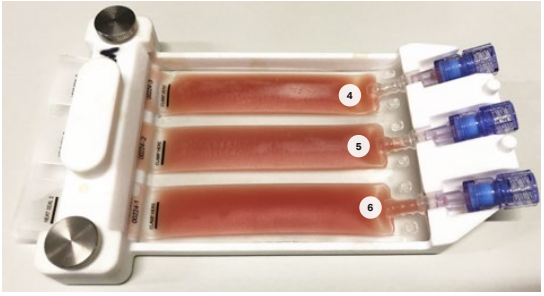


(D) **Omics pouch and clamp assembly for tissue disaggregation**

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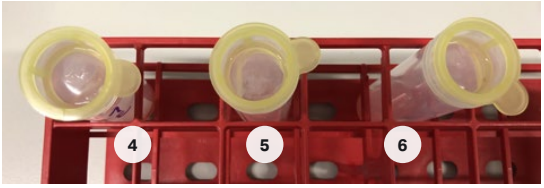


Fig 2. Tissue dissociation workflow for the gentleMACS and VIA Extractor™ tissue disaggregator:

A) Complete workflow for gentleMACS dissociation method.

B) Complete workflow for VIA Extractor™ tissue disaggregator dissociation method.

C) gentleMACS C tubes were used (C-i) along with MACSmix Tube Rotor (C-ii) to disaggregate liver tissue. Single-cell suspension was obtained (C-iii) after the 30 minute incubation. Cells were passed over pre-wet 100 µM cell strainers (C-iv). Cells were then passed over pre-wet 100 µM cell strainers (D-iii) to filter out any undigested tissue material and debris.

D) VIA Extractor™ tissue disaggregator assembly for tissue disaggregation. (D-i) Samples with enzyme mix in Omics assembly. (D-ii) Samples following 10 minutes disaggregation at 37°C. (D-iii) Cells were then passed over pre-wet 100 µM cell strainers (D-iii) to filter out any undigested tissue material and debris.

Results

The VIA Extractor™ tissue disaggregator from Cytiva produced higher yields of single cells in suspension with better viability when compared to the gentleMACS dissociator.

Cell counts were consistently higher for samples generated using the VIA Extractor™ tissue disaggregator compared with gentleMACS. Cell viability was also higher on samples prepared using VIA Extractor™ tissue disaggregator (samples 4–6, 72% ± 7% cell viability) versus gentleMACS cell dissociator (samples 1–3, 52% ± 3.6% cell viability) (Fig 2). This reduced efficiency is consistent with the observation that a considerable amount of undigested liver tissue debris remained on the cell strainer following use of the gentleMACS system (Fig 2).

Following dissociation, all samples were counted using automated TC20 cell counter and confirmed with the Haemocytometer. Results are shown in Table 4. Samples 1–3 were obtained using the gentleMACS dissociator while samples 4–6 were obtained using VIA Extractor™ tissue disaggregator. Viability and yield obtained using the VIA Extractor™ tissue disaggregator were significantly higher than for the gentleMACS. Cell counts as determined after sequencing from Cell Ranger software were significantly higher when using the VIA Extractor™ tissue disaggregator. Note that all samples were normalized to 800 cells/µL prior to partitioning, suggesting that more cells survive the process when prepared using the VIA Extractor™ tissue disaggregator.

Table 4. Cell viability and yield

Sample	Method	Total yield (cells/mL)	Live count (cells/mL)	Viability (%)	Haemocytometer count (cells/mL)
1	gentleMACS	4.64×10^6	2.59×10^6	56	2.2×10^6
2	gentleMACS	6.13×10^6	3.01×10^6	49	2.9×10^6
3	gentleMACS	5.35×10^6	2.75×10^6	51	2.3×10^6
4	VIA Extractor™ tissue disaggregator	9.94×10^6	5.30×10^6	77	4.9×10^6
5	VIA Extractor™ tissue disaggregator	1.07×10^7	5.81×10^6	75	5.1×10^6
6	VIA Extractor™ tissue disaggregator	1.71×10^7	6.71×10^6	64	5.9×10^6

Single-cell sequencing data generated via 10x Chromium platform reveals that single-cells suspension preparations obtained using VIA Extractor™ tissue disaggregator produce more cells and lower levels of cellular stress than the gentleMACS.

To better understand the impact of the two tissue dissociation methods (VIA Extractor™ tissue disaggregator vs gentleMACS) on the single-cell suspensions, single cell RNA sequencing was carried out on all samples described above. The aim was to investigate any transcriptional alterations induced on the cells by the different dissociation methods.

Although all samples were adjusted to 800 cells/µL prior to loading onto the Chromium controller, post sequencing quality control checks using Cell Ranger software revealed that the estimated number of recovered cells was higher in samples processed using the VIA Extractor™ tissue disaggregator (1086.3 ± 69.6 cells) compared to gentleMACS (479.3 ± 80.8 cells) (Fig 3). In addition, the fraction of reads in cells, was higher in samples generated using the VIA Extractor™ tissue disaggregator (59.7% ± 5.7%) compared to gentleMACS (49.8% ± 7.4%). Importantly, the fraction of reads in cells obtained using VIA Extractor™ tissue disaggregator is similar to the 58.4% reported for human liver data deposited on 10xQC website (https://10xqc.com/). Overall, these sequencing results indicate that the quality of the cells generated using the VIA Extractor™ tissue disaggregator is superior to gentleMACS for both viability of cells and number of cells recovered.

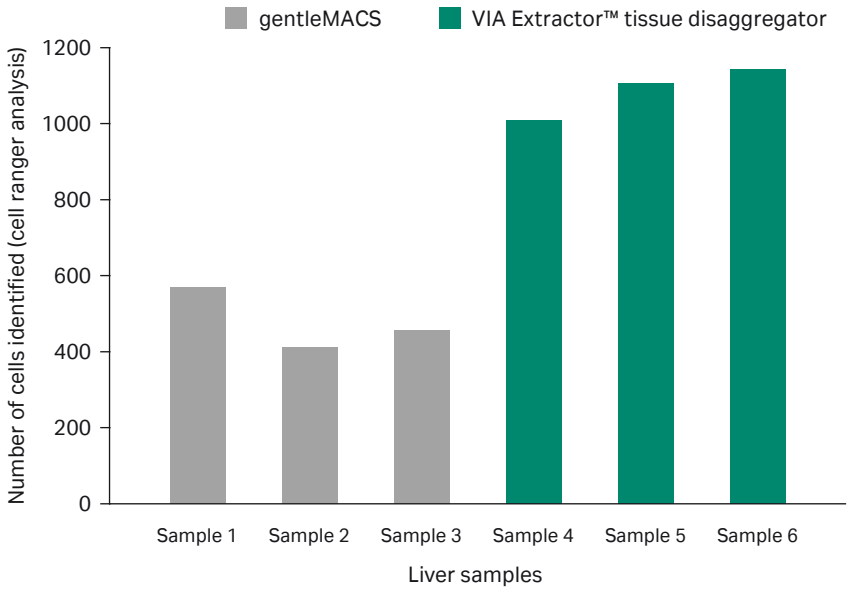


Fig 3. Cell viability. Cell counts as determined after sequencing from Cell Ranger software were significantly higher when using the VIA Extractor™ tissue disaggregator. Note that all samples were normalized to 800 cells/µL prior to partitioning, suggesting that more cells survive the process when prepared using the VIA Extractor™ tissue disaggregator.

Further analysis of the data indicates that the samples were not overly enriched for mitochondrial transcripts, suggesting that overall, the sequenced cells were viable and not overtly stressed or dying (Fig 4).

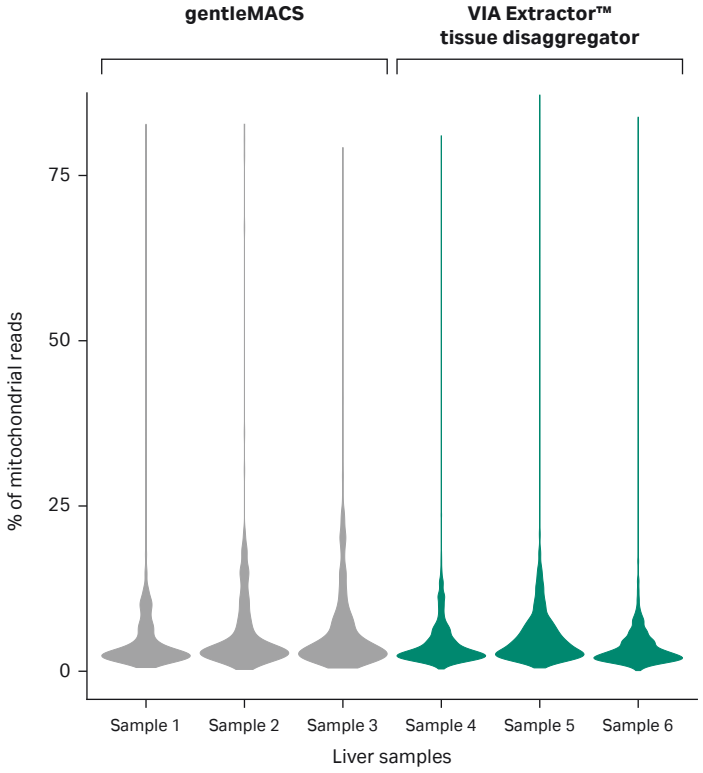


Fig 4. Distribution of percentage of mitochondrial transcripts per sample. The percentage distribution of mitochondrial content in samples confirms that the cells were viable when subjected to 10x Genomics workflow. Samples 1–3 were processed using gentleMACS, while samples 4–6 were processed using the VIA Extractor™ tissue disaggregator.

Although the same clusters and cell types were identified using both tissue dissociation methods, on average, twice as many cells from the VIA Extractor™ tissue disaggregator sample set were represented in the analysis compared with gentleMACS (Fig 5).

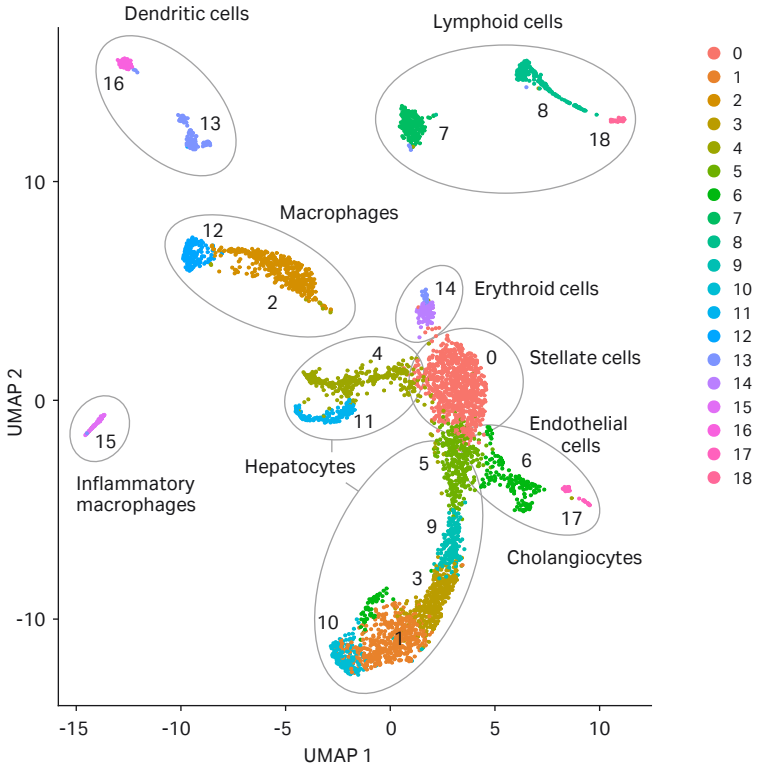


Fig 5. UMAP clustering of cell samples. Following sequencing, 19 clusters were automatically generated for both sample sets by Seurat analysis and cell types assigned

Interestingly, deeper analyses of the data (Fig 6) revealed that the proportion of cells within each cluster was consistently higher in the sample generated using the VIA Extractor™ tissue disaggregator, with the exception of cluster 10, where the proportion of cells was higher in samples generated by gentleMACS (Fig 6B).

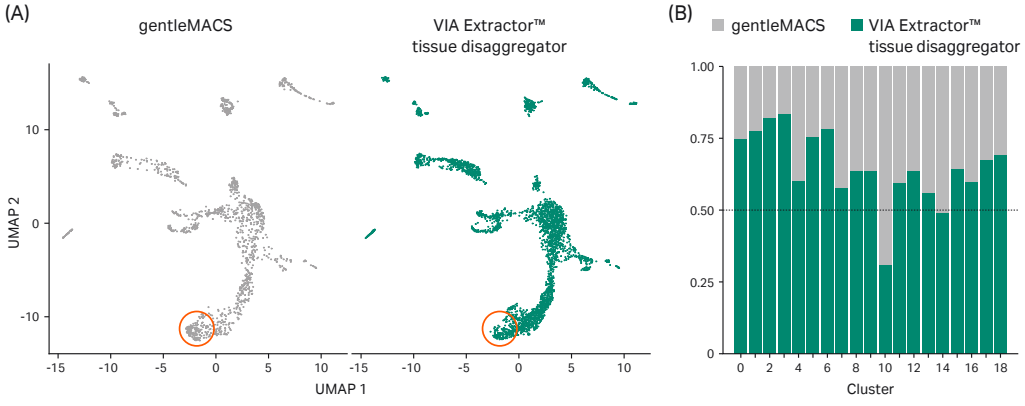


Fig 6. UMAP clustering of cells and differential abundance of cells. (A) UMAP clustering of all cells by sample set (gentleMACS and VIA Extractor™ tissue disaggregator). While the clustering is similar, there are significantly more cells in each cluster from tissue processed using the VIA Extractor™ tissue disaggregator. (B) Proportional representation of cells from both sample sets in each of the clusters identified in Figure 5 indicate that for samples processed using the VIA Extractor™ tissue disaggregator, there are significantly more cells present in most clusters with the exception of cluster 10, which is higher for samples processed using the gentleMACS system.

To identify the nature of the cells overrepresented in cluster 10 of the samples processed using gentleMACS, a gene ontology (GO) analysis was performed on the top 20 most highly expressed genes that were detected in this cluster. This analysis revealed that the cells in cluster 10 were highly enriched in active biological processes that are associated with cellular stress and cell death (Fig 7). This observation is consistent with the fact that fewer cells passed the threshold for number of reads per cell as per 10x Genomics' recommendation than for the VIA Extractor™ tissue disaggregator. This result suggests that the single cells generated by gentleMACS are more fragile and are prone to breaking before or during the processing the sample for sequencing.

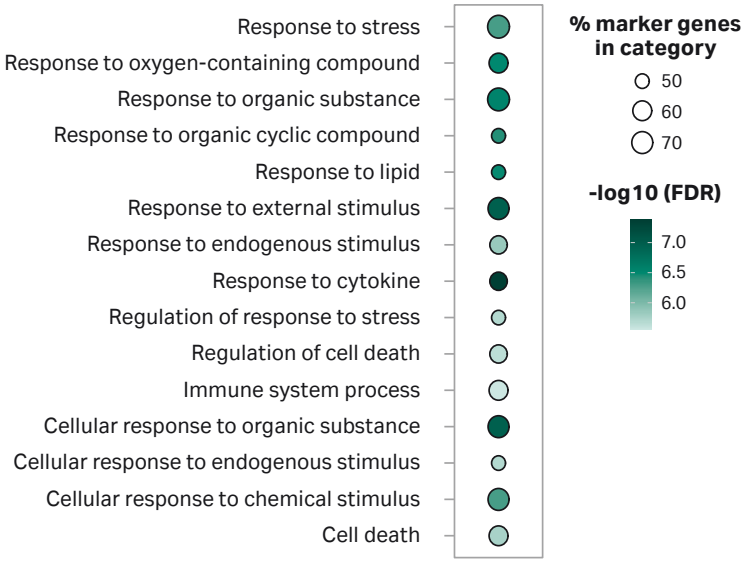


Fig 7. Gene ontology analysis for the top 20 gene markers detected in cells within cluster 10. The list of genes was analyzed using ShinyGO v0.61, which provides a list of functional categories and its respective enrichment FDRs (2). The results were plotted in R (v4.0.2) using a custom script. The top 15 more significant functional categories are shown. The percentage of marker genes in a category represents the number of genes from the top 20 gene list found to belong to any individual category.

Conclusion

Single-cell RNA sequencing methodologies are providing deep insights into the roles of individual cells in the context of tissues, organs, and even whole organisms, which helps to unravel the molecular networks underlying both embryonic development and disease. The first step in many such workflows is the dissociation of tissue samples into single-cell suspensions prior to sequencing. It is well understood that the health and viability of cells following dissociation can be key to achieving the best possible results, so choosing the lowest impact technology for generation of single-cell suspensions should be a key consideration when setting up any single-cell omics workflow. Data from this analysis showed that the VIA Extractor™ tissue disaggregator from Cytiva provided a higher yield of viable mouse liver cells in a significantly shorter time period when compared to the gentleMACS dissociator. This result is important for reducing process bias. UMAP clustering was similar between systems but showed that twice as many cells were represented in the sequencing data across nearly all clusters in single-cell suspensions generated using the VIA Extractor™ tissue disaggregator. Further gene ontology analysis indicated the presence of significantly elevated numbers of cells in the gentleMACS samples that displayed transcription profiles associated with cell fragility.

References

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2. Ge SX, Jung D, Yao R. ShinyGO: a graphical gene-set enrichment tool for animals and plants. *Bioinformatics*. 2020 Apr 15;36(8):2628-2629. doi: 10.1093/bioinformatics/btz931

Further information

Read more about single-cell sequencing and the VIA Extractor™ tissue disaggregator in the following resources:

- Whitepaper: Single-cell sequencing: the challenges and opportunities
- Blog: Single-cell RNA-seq and cold tissue dissociation
- Webpage: Request a demo of VIA Extractor
- Video: VIA Extractor™ tissue disaggregator 3D Animation
- Video: VIA Extractor™ lab-based video

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