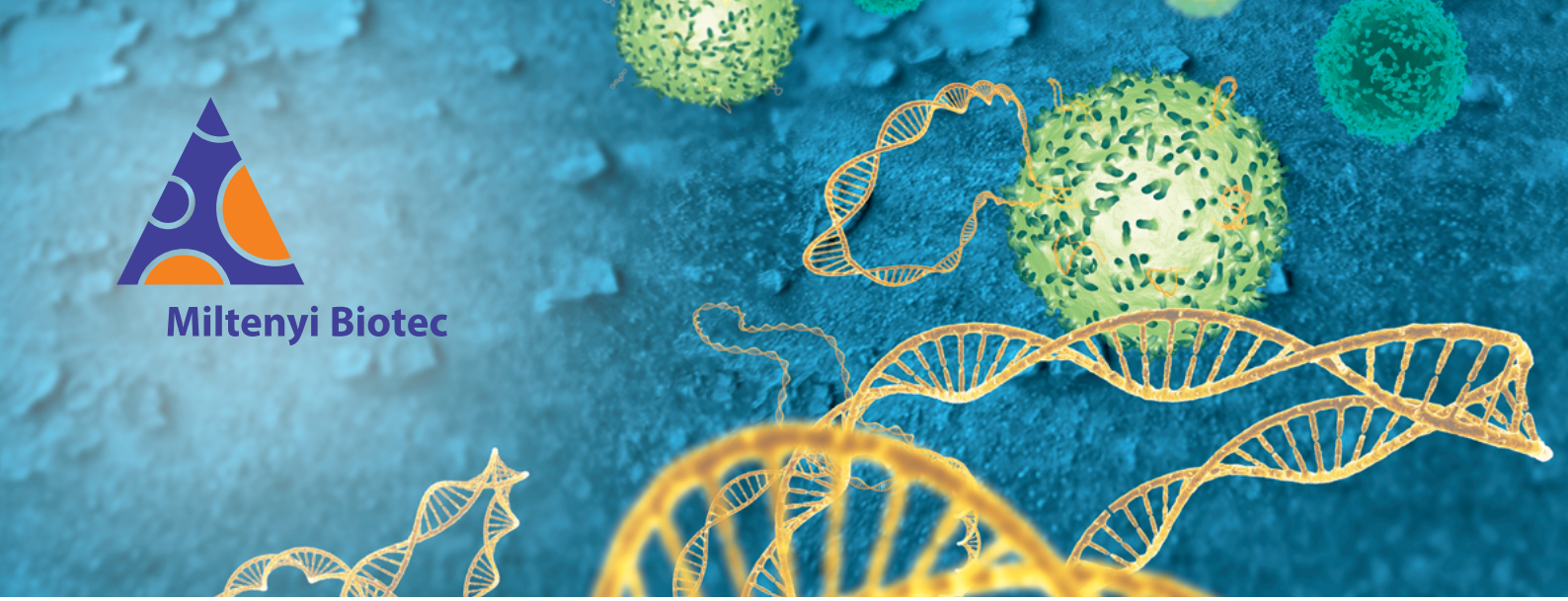

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Optimized nuclei preparation workflow for genomic applications

Background

Nuclei preparation is a crucial step for downstream genomics applications such as single-nucleus RNA sequencing (snRNA-seq), and often requires high quality and purity of nuclei to achieve reproducible results. SnRNA-seq offers numerous advantages over single-cell RNA-sequencing (scRNA-seq), including the ability to study difficult-to-isolate cell types, overcome cell-size limitations, facilitate frozen-tissue analysis, and access the nuclear transcriptome to provide valuable insights into transcriptional regulation and splicing patterns. To meet quality standards and obtain reliable results for snRNA-seq, an optimized workflow for the preparation of nuclei is required.

Automating nuclei extraction with gentleMACS™ Tissue Dissociators enables high-throughput extraction without requiring extensive hands-on time. Subsequent purification of the nucleus suspension is essential and can be accomplished in as little as 40 minutes with MACS® Technology, making the entire preparation process as simple and fast as possible.

This application note presents a comprehensive workflow for preparing nuclei from snap-frozen tissue samples, including human ovarian carcinoma (human OvCa) as well as mouse heart, liver, and brain samples. An essential factor that can affect subsequent enrichment and analysis is the correct nuclei count. In terms of sequencing, accurate counting not only determines the exact number of nuclei available, but also helps to achieve a balanced representation of different cell types within the sample.

Any inaccuracies in counting can lead to biased results, misinterpretations, or the exclusion of rare cell populations, which can significantly compromise the biological insights gained from snRNA-seq experiments. To mitigate the potential pitfalls of inaccurate counting, it is recommended to employ multiple counting methods, such as flow cytometry-based and fluorescence image-based techniques. This multifaceted approach enhances the robustness of the counting process and helps validate the accuracy of the results.

In addition, ensuring the purity of the nuclei is essential for snRNA-seq for several reasons. It ensures biologically relevant results by preventing contamination from foreign RNA sources, maintains data integrity by reducing imprecision, and improves resource efficiency by reducing the sequencing depth required for meaningful results. This application note demonstrates how to optimize nuclei preparation, perform accurate counting, and thus obtain reliable and reproducible snRNA-seq data.

Materials and methods

Nuclei extraction and enrichment

Nuclei were extracted from three samples of 50 mg human OvCa samples, whole mouse hearts, 75 mg mouse liver, and 50 mg mouse brain samples in biological triplicates using Nuclei Extraction Buffer with a gentleMACS C Tube on a gentleMACS Octo Dissociator with Heaters, according to the manufacturer's instructions. The temperature of the nucleus suspension was kept cold during the extraction process using the gentleMACS Octo Coolers. After extraction, 1×10^6 nuclei from each sample were used for further enrichment by magnetic separation with Anti-Nucleus MicroBeads on a QuadroMACS™ Separator, according to the manufacturer's instructions.*

Nuclei counting

Quantification of nuclei was performed first after extracting and second after enriching the nuclei. Analysis of the nucleus count was conducted by flow cytometry using the MACSQuant® Analyzer 10 utilizing DAPI as the nuclei staining agent. Subsequent data analysis was carried out using MACSQuantify™ Software. In parallel, single-nuclei suspensions were stained using the acridine orange (AO)/propidium iodide (PI) Viability Assay (DeNovix®) in order to differentiate between nuclei, intact cells, and debris. Nuclei count was then determined using the CellDrop™ FL Automated Cell Counter (DeNovix), according to the manufacturer's instructions.

Fine-tuning the nuclei count for nuclei with aberrant shape
Adjusting protocol settings for improved nuclei counts in specific tissues with the CellDrop FL Automated Cell Counter (CellDrop FL) relies on the user's expertise to optimize the settings for different sample types. Lowering the maximum diameter threshold helps separate nuclei that are counted as a single large object, while increasing the threshold prevents single large objects from being inaccurately counted. Fluorescence thresholds reduce interference from background signals.

The so-called "irregular cell mode" accommodates non-spherical nuclei, such as those found in smooth muscle and cardiac cells as well as in binucleated cells that are regularly present in cancer samples. Once this mode is set, diameter adjustments can be performed in real time for each tissue and saved for future nuclei counts. Ensuring appropriate fluorescence threshold settings is also an important consideration depending on media/extraction or if high exposures are required. For more information, please visit the DeNovix website.

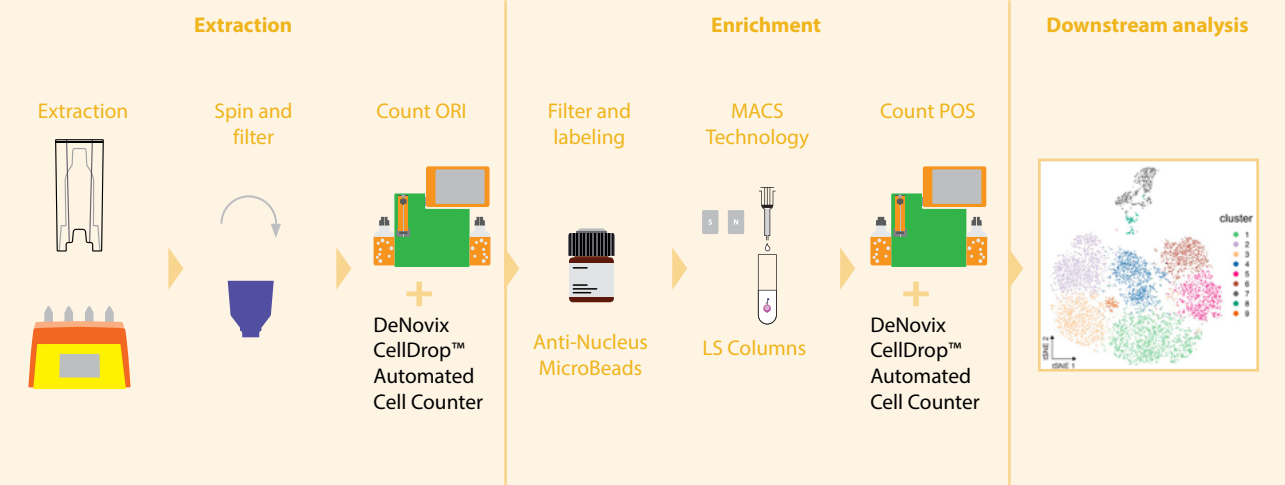

Figure 1: Nuclei preparation workflow overview. Generation of single-nucleus suspensions from tissue for snRNA-seq analysis, including automated extraction on the gentleMACS Octo Dissociator with Heaters and enrichment with Anti-Nucleus MicroBeads based on MACS Technology. Ori: original fraction, POS: positive fraction.

Fluorescence imaging of nuclei

For fluorescence microscopy analysis, the brain nuclei were stained after enrichment using DRAQ5™. Subsequently, these stained nuclei were examined using the laser-scanning microscope LSM 710 (ZEISS®) at 63× magnification using a Plan-Apochromat 63×/1.4 Oil DICII objective (ZEISS®). This configuration allowed in-depth inspection of nuclear integrity.

Results

Efficient and automated nuclei extraction from different tissue types
Automated nuclei extraction for each tissue type was performed in triplicates, each sample of the same weight. The total extraction volume for each sample was 4 mL after rinsing the gentleMACS C Tube with an additional 2 mL of Nuclei Extraction Buffer to maximize the recovery of nuclei (as described in the Nuclei Extraction Buffer data sheet). Following extraction, nuclei quantification was conducted on the MACSQuant Analyzer 10 utilizing DAPI staining (fig 2).

In detail, extraction of human OvCa samples obtained from 50 mg tissue fragments yielded an average number of 1.46×10^5 nuclei/mg. Whole mouse heart samples yielded an average of 0.48×10^5 nuclei/mg. For liver samples weighing 75 mg, the average nuclei yield was 2.26×10^5 nuclei/mg, while mouse brain samples weighing 50 mg, the average yield of nuclei was 2.36×10^5 nuclei/mg. Overall, the individual nuclei concentrations of the biological replicates demonstrated a high level of reproducibility.

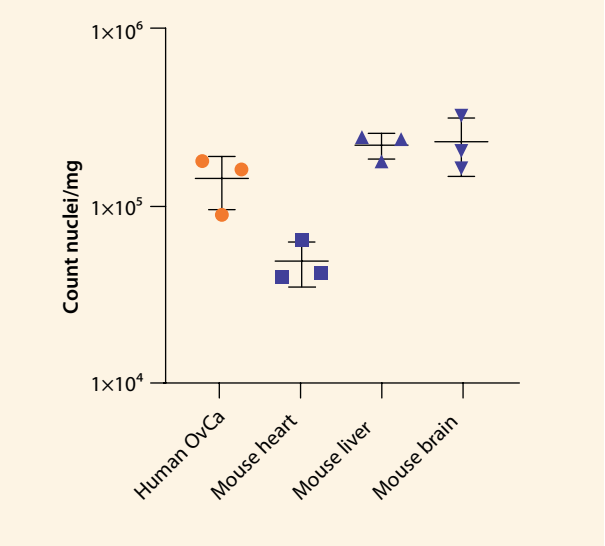

Figure 2: Preparation of single-nucleus suspensions from different tissues. The figure shows the numbers of extracted nuclei per mg of dissociated human OvCa, mouse heart, mouse liver, and mouse brain, as indicated in the figure legend. Single-nucleus suspensions were obtained from frozen tissue samples by using the Nuclei Extraction Buffer in combination with the gentleMACS Octo Dissociator with Heaters and installed frozen gentleMACS Octo Coolers. Nuclei were stained with DAPI and analyzed by flow cytometry using the MACSQuant Analyzer 10.

Comparable nuclei counts between MACSQuant Analyzer and CellDrop FL for increased reliability

To validate the nuclei concentration values, a comparative analysis was conducted between the cell counter (CellDrop FL) and the MACSQuant Analyzer 10, both immediately after nuclei extraction (fig. 3A) and after nuclei enrichment (fig. 3B). To better illustrate the comparison, nuclei/mL concentration values were assessed. Strikingly, an initial observation reveals a close alignment in the number of nuclei concentration recorded across all tissue types (fig. 3A).

In particular, human OvCa samples exhibited a notable prevalence of nuclei from binucleated cells, a phenomenon commonly seen in tumor samples, which appeared as a distinct population in the flow cytometer dot plot with increased DAPI signal and interconnected nuclei in the fluorescence microscopy image. Fine tuning of the settings on the CellDrop FL was necessary to accurately quantify these binucleated cells as described in the method section.

Furthermore, due to the non-uniform, oval/oblong morphology of nuclei in heart and muscle tissues, specific adjustments in the cell counter were also essential to determine accurate nuclei concentration (see methods section). Subsequently, 1×10^6 nuclei from each sample were further enriched by MACS Technology using Anti-Nucleus MicroBeads and the numbers were re-evaluated after elution of the positive fraction (POS) (fig. 3B). Again, no significant differences were observed between the results of the two counting methods, demonstrating their high comparability.

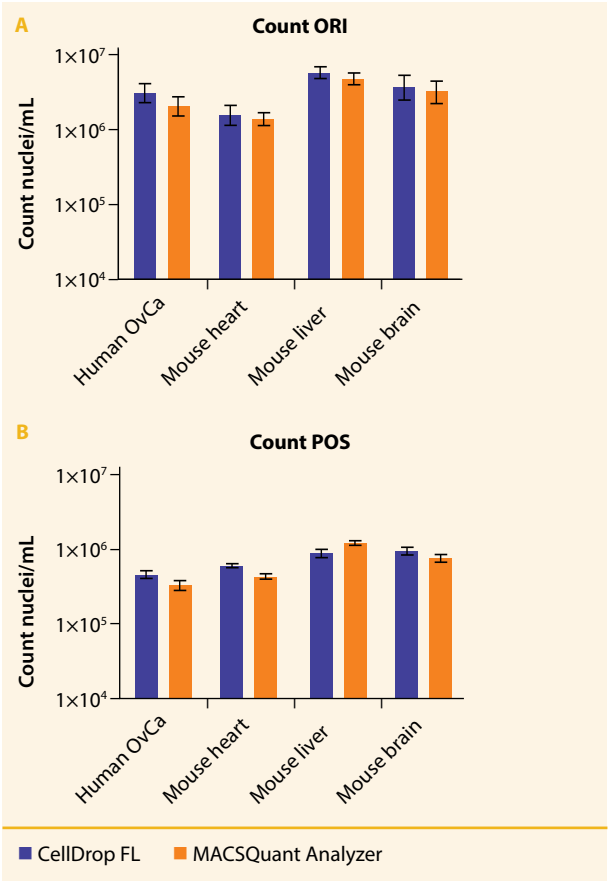

Figure 3: Comparison of nuclei counts. Nuclei quantification was performed using both the MACSQuant Analyzer 10 with DAPI staining and the CellDrop FL Automated Cell Counter (CellDrop FL) with AO/PI staining in two stages: (A) after extraction: ORI, and (B) after enrichment: POS.

Anti-Nucleus MicroBeads significantly increased nuclei purity in all samples

Next, the purity of nuclei from each sample was determined using the MACSQuant Analyzer 10 by measuring the DAPI-positive events before and after enrichment (fig. 4A). For human OvCa samples, purity was already at a high level of approximately 80% immediately after extraction, and enrichment resulted in an increase in purity to approximately 90%. Mouse heart and mouse liver samples showed lower purity after extraction, both at around 60%.

Further enrichment of both mouse heart and liver samples resulted in a significant increase in purity to around 90%. High amounts of debris were found in mouse brain samples with a very low nuclei purity of approximately 6%. Nucleus enrichment was able to significantly increase the purity of the mouse brain nucleus suspension to over 90%, demonstrating the strength of Anti-Nucleus MicroBeads in bringing samples with high amounts of debris to maximum purity in a relatively short period of time.

While purity was quantified using the flow cytometer, the images from the CellDrop instrument were used as a visual indication of sample quality (fig. 4B). Here, we observed both debris and residual viable cells present in the original fraction (ORI) and clean samples with only nuclei obtained in the enriched fraction (POS), confirming the purity results previously obtained with the MACSQuant Analyzer.

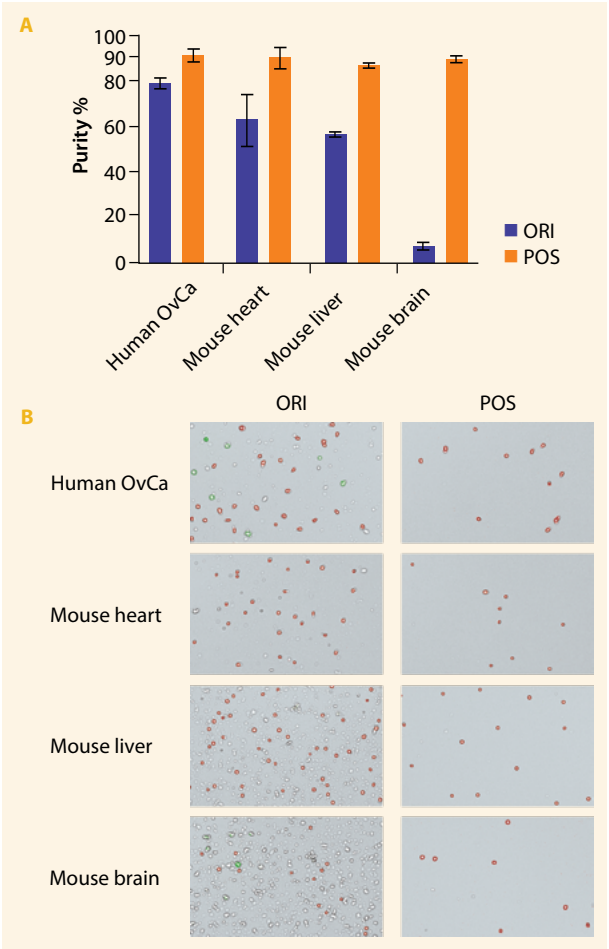

Figure 4: Purity assessment after enrichment with Anti-Nucleus MicroBeads using MACS Technology. (A) The percentage of nuclei purity, including both ORI and POS levels, measured with the MACSQuant Analyzer 10 and visualized with DAPI staining. (B) Cropped image regions from the CellDrop FL to illustrate changes in ORI and POS samples. The nuclei, shown by red PI staining, represent DNA, while viable cells are highlighted in green with AO staining. Unstained particles are identified as debris.

Enriched nuclei demonstrate high integrity as seen in microscopic images

Visual validation of nuclei integrity is highly recommended when downstream experiments are planned that require high-quality assessment of nuclei, such as snRNA-seq. To demonstrate the integrity of enriched brain nuclei samples, fluorescence imaging was performed at 63× magnification (fig. 5). The results demonstrate clean samples, with the lack-of-debris particles confirming the high levels of purity determined by flow cytometry. In addition, the nuclei have a round appearance and sharp borders, highlighting the integrity of the nuclei, as clearly seen in the enlarged picture on the right.

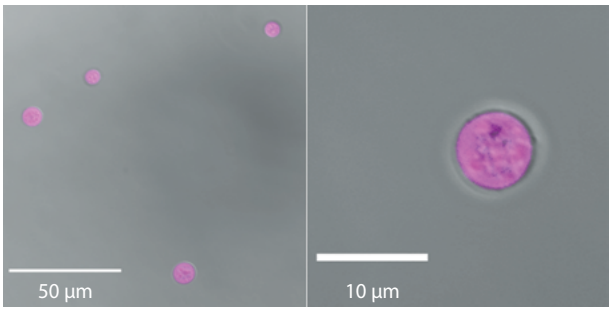

Figure 5: Single-nuclei suspension from enriched mouse brain nuclei. Single nuclei were enriched via MACS Technology using Anti-Nucleus MicroBeads. Immediately after the nuclei enrichment, the nuclei were stained using DRAQ5 Staining Solution. The image shows an overlay of DRAQ5 (pink) and brightfield obtained with a LSM 710 microscope at 63× magnification.

LEARN MORE

See how enriched nuclei generate high-quality snRNA-seq results with the Chromium™ Single Cell 5′ v1.1 Reagent Kit (10× Genomics®) and an Illumina® System.

▸ **miltenyibiotec.com/nuclei-enrichment**

Conclusion

- Automated extraction of nuclei using Nuclei Extraction Buffer on the gentleMACS Octo Dissociator with Heaters from a variety of tissues results in a high yield of nuclei. The use of gentleMACS Octo Coolers during the process ensures the integrity of the nuclei.
- Enrichment with Anti-Nucleus MicroBeads increases the purity of all sample types, ensuring high-quality sample input for downstream genomics applications, such as single-nucleus RNA sequencing.
- Accurate cell-counting methods, using systems such as the MACSQuant Analyzer and the DeNovix CellDrop FL, show close correlation in the cell concentration numbers obtained across all tissue types, ensuring accurate quantification.
- High-quality snRNA-seq results can be achieved with enriched nuclei, enhancing transcriptome confidence, improving gene counts, and reducing error susceptibility for downstream experiments.

Products	Order no.
Nuclei extraction	
Nuclei Extraction Buffer	130-128-024
gentleMACS C Tubes	130-093-237
gentleMACS Octo Dissociator with Heaters	130-096-427
gentleMACS Octo Coolers	130-130-533
MACS SmartStrainers (30 µm)	130-098-458
MACS SmartStrainers (70 µm)	130-098-462
MACS SmartStrainers (100 µm)	130-098-463
MACS BSA Stock Solution	130-091-376
Nuclei enrichment	
Anti-Nucleus MicroBeads	130-132-997
LS Columns	130-042-401
QuadroMACS Separator	130-090-976
MACS MultiStand	130-042-303
Nuclei staining	
MACSQuant Analyzer 10	130-096-343
DAPI Staining Solution	130-111-570
DRAQ5 Staining Solution	130-117-343

* RNase inhibitor (0.2 U/µL final concentration) was added to the nucleus separation buffer for mouse brain samples after the extraction procedure for subsequent enrichment and preparation of snRNA-seq.

Miltenyi Biotec

Miltenyi Biotec B.V. & Co. KG | Friedrich-Ebert-Straße 68 | 51429 Bergisch Gladbach | Germany | Phone +49 2204 8306-0 | Fax +49 2204 85197
macsde@miltenyi.com | **www.miltenyibiotec.com**

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