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A step towards enzyme-free tissue dissociation

Abstract: The future of personalized diagnostics commences on the single cell level. Even high-end technologies like Next Generation Sequencing can be improved if applied on pure single cell populations (e.g., tumor cells without contaminating stromal cells) or on a single cell level (DNA/RNA sequencing). The vast majority of these technologies need individual and preferably undistorted cells for the analytical process. Thus, decisive prerequisite for high-end analytics is to remove cells from their tissue matrix as gently as possible. This can be accomplished by an enzyme-free, fast and reproducible approach of generating pure and individual single cells from tissue samples. In this study we demonstrate the utility of a semi-automated Tissue Grinder that is compatible with standard 50 ml centrifuge tubes and standard cell strainer for mechanically, non-enzymatic and parallel processing of tissue samples. We show that without enzymatic treatment viable single-cell yields match or exceed reference enzymatic methods, while reducing processing time by at least 80%.

Keywords: Tissue Dissociation, Enzyme-free, Single Cell, Laboratory Automation

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1 Introduction

With the increasing applications of patient derived cells as a model system for research, the need for high quality cell material is continuously growing [1, 2]. The ability to extract individual cells from tissues is currently a bottleneck for cell-based diagnostic technologies and remains crucial in the areas of cancer research, emerging diagnostic methods, tissue engineering and personalised medicine approaches [3, 4]. Main challenges include the yield and quality or in other words the integrity and purity of the cells as well as the throughput and reproducibility. Tissue is typically

disintegrated by proteolytic digestion and various mechanical treatments. These conventional methods of tissue dissociation include titration, manual cutting with scalpel, micro-scissors, tissue choppers and micro fabricated filters, expose the sample at high risk of contamination [5]. However, success has been limited by long processing times, low yield and high manual workload leading to a significant increase in the overall analysis time [6]. Furthermore, enzymatic reactions can attack surface markers and cause alteration in gene expression profiles. Tissue dissociation is therefore a careful balance between disrupting the tissue while not lysing the cells [7]. To overcome these limitations, we developed a mechanical tissue dissociation system based on counter-rotating rows of grinding teeth integrated in a standard 50 ml centrifuge tube. In this study, we describe a complete workflow for enzyme-free individual processing of tissue samples resulting in optimal process parameters and a minimised processing time.

2 Tissue Grinder

Single cell analysis requires effective, sensitive, and quantitative techniques for separation and detection [5]. Therefore, a desired tissue dissociation system is robust enough to effectively singularise the tissue while maintaining cell viability and cell yield. Compatibility with commercially-available standard disposables is a prerequisite as well as reducing process time by combination of process steps and yielding an output that is simply accessible by liquid handling robotics for automated laboratory solutions. In addition, the system is applicable for multiple tissue types (structural properties and sample size). Ultimately, the system reduces the need for enzymatic digestion and processing of multiple samples independently in parallel.

2.1 Core unit of the Tissue Grinder

The resulting Tissue Grinder can be subdivided into a benchtop instrument and the actual grinding tube with the integrated core unit and cell strainer, whereby the grinding tube is designed as sterile disposable. The two-part core unit, consisting of rotor and stator inserts equipped with counter

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rotating grinding teeth (Figure 1). This results in an interlocking grinding unit with rows of teeth that can be moved against each other. The free space between the grinding teeth and their fin-shaped geometry is designed in a way that efficient extraction of viable single cells is ensured. The rotation of the grinding teeth draws the tissue sample into the free space applying adequate amounts of shearing and milling force to gently isolate single cell from the tissue. The direction of rotation can be selected individually and determines the type of tissue dissociation (shearing and milling and combinations).

Figure 1: Core unit of the Tissue Grinder. (a) Core unit of the Tissue Grinder utilizes a rotor and stator insert equipped with counter rotating grinding teeth. (b) Picture of fabricated grinding inserts. Clockwise is a cutting (shearing) process, counter clockwise is a grinding/friction (milling) motion. Due to the fin-shaped geometry by changing the direction of rotation different forces can be applied resulting in a wide range of tissue structures which can be processed. (c) Schematic showing the dimensions of the space between the grinding teeth.

2.2 Workflow of the Tissue Grinder

The Tissue Grinder consists of different components, which are integrated in a disposable 50 ml centrifuge tube (Figure 2). The loading sample size depends on the type of the examined tissue, to maintain the optimum working range of 50 to 300 mg tissue is recommended. The Tissue Grinder is constructed in such a way that the filtration of the dissociated single cells from the remaining tissue can be carried out immediately after the dissociation without further preparation and pipetting steps. The centrifuge tube protects and encapsulates the tissue sample during dissociation and collects the single cell suspension after centrifugation. By the integration of the cell strainer, tissue dissociation and sample clearing are combined in one process step, minimizing the risk of contamination by reduced exposure time to non-sterile conditions and to ensure reliable generation of single cell suspensions.

Figure 2: Tissue dissociation workflow and benchtop device. (a) The rotor insert is positioned in the lid of the centrifuge tube. (b) Tissue sample is placed into the free space between the rotor grinding teeth. (c) The stator insert of the grinding unit is inserted in a standard nylon cell strainer. (d) In the next preparatory step, the centrifuge tube is placed on the lid and screwed to it. (e) Grinding tube and benchtop unit are brought together by a form-fit connection. A geared motor rotates an axis which is connected to the rotor insert. The direction of rotation can be set to clockwise or counterclockwise.

The process parameters time, direction of rotation, acceleration, speed and the number of process steps can be adjusted by the operator when creating an individualised protocol for a specific type of tissue sample, in order to allow for rapid optimisation of the individual dissociation protocol. Table 1 provides an overview of the process parameters and device specifications of the Tissue Grinder.

Table 1: Overview Tissue Grinder specifications

Specification	
Throughput	4 tubes in parallel
Cell strainer (nylon)	40, 70, 100 μm mesh size
Platform Disposable	Standard 50 ml conical centrifuge tubes
Loading capacity/tube	50-300 mg tissue plus 500 μl Buffer
Motor power rating	2.5 W
Torque	0.6 Nm
Grinding speed	Working range 30-100 rpm
Rotation modus	Shearing/milling and combinations

To demonstrate the utility of our instrument, we monitored cells viability, yield and dissociation grade of murine liver samples. Liver tissues represent samples that will be used in future applications, and the resulting cell suspensions can be directly assayed for quality. The commercially available tissue processing system gentleMACS® from Miltenyi Biotec was used as a benchmark.

3 Materials and Methods

3.1 Tissue sourcing and dissociation

Freshly isolated murine liver tissue was stored in RPMI media and placed on ice for shipping. Samples were processed within 24 h of harvesting to ensure maximum cell viability. Immediately after harvesting, tissue samples were cut into 0.5 cm³ pieces with a scalpel and weighed.

The specially constructed Tissue Grinder shown in Figure 2 and the gentleMACS® Dissociator were employed in this research. Rotor and stator inserts were manufactured from polycarbonate (PC) to meet requirements for chemical and mechanical stability as well as cost requirements for a single use disposables. C-Tubes were used for the gentleMACS®.

3.2 Methods

The experimental dissociation protocol for the Tissue Grinder was determined by carrying out preliminary test with varying process parameters. 110–150 mg of tissue sample was used per tube. After completing the dissociation protocol, the grinding tubes were centrifuged at 300×g for 5 min with 70 µm cell strainer. The sample preparation and processing for the gentleMACS® was performed according to the commercially available liver enzyme kit (Order No.:130-105-807) from Miltenyi Biotec. Tissue samples for the Tissue Grinder were processed without enzyme (TG). GentleMACS® tissue samples were prepared for one series of experiments with enzymatic (GM+) and one without enzymatic pretreatment (GM-).

Cell yield was quantified by the number of viable cells produced by Tissue Grinder and gentleMACS® via standard trypan blue dye-exclusion method. To examine the impact of mechanical dissociation on cell viability, we next performed a propidium iodide (PI) exclusion assay to identify viable or dead cells. Cell scattering and fluorescence signal intensities were acquired using an FACS Canto II (Becton Dickinson), and analysed using FlowJo software (Tree Star). After evaluating for cellular viability and yield, the single-cell suspension was inspected for the dissociation grade, defined by the difference between the input weight and the remaining tissue in the tubes after processing.

$$\text{Dissociation grade} = \frac{\text{Tissue}_{\text{input}} - \text{Tissue}_{\text{processed}}}{\text{Tissue}_{\text{remaining}}} \quad (1)$$

Data are represented as the mean ± standard error. Error bars represent the standard error from at least three independent experiments.

4 Results

For a proof-of-concept we tested the grinding tubes using freshly resected murine liver samples. Dissociation device experiments were conducted as described. To determine the advantages of non-enzymatic cell dissociation by the Tissue Grinder compared with standard enzymatic treatment by the gentleMACS®, we performed cell counts, cell viability and dissociation grade determination. Results are shown in Figure 4 and 5. As can be seen, Tissue Grinder processing without enzymatic sample treatment largely matched or exceeded numbers of viable cells and yield in considerably shorter processing times. The enzymatic pre-treatment showed advantages in the dissociation grade of the tissue sample. Obviously the enzymatic treatment caused a loosening of the tissue structure and thus a more complete processing. For the study here, the dissociation by the Tissue Grinder was completed in less than two minutes and offered a substantial time saving when compared to the gentleMACS® method (ca. 60 min), particularly when parallel processing of multiple samples is considered.

Figure 3: Cell yield and dissociation grade after dissociation of liver tissues by Tissue Grinder and gentleMACS®. A) Cell yields following dissociation of liver tissue were compared to GM-, GM+ and TG. B) Dissociation grade was quantified from remaining tissue for GM-, GM+ and TG.

We could observe that due to the direct centrifugation of the grinding tubes, the output single-cell suspension is largely free of debris and can be easily handled by robotic liquid

handling systems. Thus, the Tissue Grinder significantly improves dissociation efficiency and shortens processing time.

Figure 4: Further characterization of the single-cell suspension from the different dissociation procedures was achieved using flow cytometry.

5 Conclusion and Outlook

In this paper, we present a novel enzyme-free approach for mechanically dissociating of tissue samples into viable single-cell suspensions. Liver is generally considered to be among the easiest tissues to dissociate, but hepatocytes are well known to be fragile [5]. Therefore this method is suitable, in particular as a model system, to prove the gentle tissue dissociation of an enzyme-free approach. Using this model we demonstrated that the Tissue Grinder produces comparable cell yield and cell viabilities, without enzymatic treatment, when compared to the gentleMACS® system with enzymatic treatment. Furthermore the results show a reduction of the overall process time for the tissue dissociation. Due to this concept, a high level and high throughput of enzyme-free single cell suspension could be generated for sophisticated molecular analysis. High-quality single-cells are fundamental for analysing tumour heterogeneity and future personalized approaches in medicine.

Other types of tissue currently are under investigation, optimization will focus on torque sensor integration, which

will provides continuous information about the structural integrity of the tissue sample during the dissociation process enabling precisely controlled, rapid and enzyme-free tissue dissociation.

Author Statement

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