Whole genome amplification of single epithelial cells dissociated from snap-frozen tissue samples in microfluidic platform

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ABSTRACT

Single cell sequencing is a technology capable of analyzing the genome of a single cell within a population. This technology is mostly integrated with microfluidics for precise cell manipulation and fluid handling. So far, most of the microfluidic-based single cell genomic studies have been focused on lab-cultured species or cell lines that are relatively easy to handle following standard microfluidic-based protocols without additional adjustments. The major challenges for performing single cell sequencing on clinical samples is the complex nature of the samples which requires additional sample processing steps to obtain intact single cells of interest without using amplification-inhibitive agents. Fluorescent-activated cell sorting is a common option to obtain single cells from clinical samples for single cell applications but requires >100 000 viable cells in suspension and the need for specialized laboratory and personnel. In this work, we present a protocol that can be used to obtain intact epithelial cells from snap-frozen postsurgical human endometrial tissues for single cell whole genome amplification. Our protocol includes sample thawing, cell dissociation, and labeling for genome amplification of targeted cells. Between 80% and 100% of single cell replicates lead to >25 ng of DNA after amplification with no measurable contamination, sufficient for downstream sequencing.

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INTRODUCTION

Single cell whole genome sequencing (SC-WGS) is a powerful tool for investigating the evolution, genetic diversity, and heterogeneity of complex biological samples and is paramount to our understanding in disease progression.^{1–3} This technology offers the capability of finding out the genomic mutations of a single cell within a population, which is often neglected in conventional approaches such as metagenomics.^{4,5} For this reason, SC-WGS is starting to shed new light on critical questions in human disease that are challenging to address by conducting conventional bulk analyses. This brings forth new prospects such as evaluating the role of genetic mosaicism⁶ in critical diseases.^{7–9}

Typical SC-WGS processes include single cell isolation, lysis, and amplification from femto to picograms of genomic DNA to reach the quantity sufficient for standard downstream library preparation and sequencing (>25 ng).^{10,11} Microfluidic platforms are often

used for single cell whole genome amplification (SC-WGA) due to their unique ability of handling nanoliters of fluid with precise control,^{12–18} thus allowing for the isolation of single cells into individual compartments for lysis and genome amplification.^{19,20} Multiple displacement amplification (MDA)²¹ has been a popular option for SC-WGA in microfluidic platforms.^{22–24} It is based on ϕ 29 DNA polymerase and random primers to copy template DNA with high fidelity and lower error rates using simple procedures compatible with microfluidic platforms.^{25–27}

So far, most of the microfluidic-based SC-WGA applications have been focused on lab-cultured species or cell lines that are relatively easy to handle following standard microfluidic-based protocols without additional efforts.^{3,20} These efforts are important technological development; however, SC-WGA of cells from clinical samples such as biopsies and surgically removed tissues and tumors is still rare due to practical concerns including the complex nature of these samples. Besides, it is often logistically challenging to

perform SC-WGS on these fresh samples immediately after collection as the multiple specialized laboratory facilities involved are in different locations.²⁸ Routinely used clinical sample preservation methods such as formalin fixation and paraffin embedment preserve good cell integrity but largely limits MDA-based amplification.²⁹

Snap-freezing is a common alternative for preserving clinical samples especially postsurgical samples due to the practicality of using dry ice or liquid nitrogen and required buffers in the operating room.^{30,31} Tissue samples collected for genomic analyses are typically snap-frozen without buffer or in Tris-EDTA (TE) buffer and stored at -80°C . Standard sample handling downstream such as freeze-thaw could compromise cell integrity which is critical for single cell analyses. It is worthwhile to note that the conventional cell dissociation methods have been effective in dissociating single cells from a variety of freshly collected human and animal tissue samples.^{32–35} However, single cell dissociation from snap-frozen tissue samples remains a challenge, hindering the efforts of performing single cell genomic analyses on clinical samples collected and stored in a standard manner. To the best of our knowledge, no protocol exists that allows the single cell dissociation from these samples while preserving cell integrity.

Fluorescent-activated cell sorting (FACS)^{36,37} can be an option for single cell isolation and sorting but relies on specific handling procedures and buffers to maintain cell integrity and requires a large number of dissociated single cells ($\sim 10^6$ – 10^7 cells/ml). It is not always possible to have an abundance of viable cells in the clinical tissue samples; thus, FACS is not an ideal approach for dissociation and sorting of target single cells from clinical tissues for genomic studies. Droplet microfluidics are becoming popular due to their capabilities of cell encapsulation in a high-throughput manner.^{13,19} However, the number of cells encapsulated in each droplet matches the Poisson distribution, thus reducing the probabilities of ensuring single cell capture. Using micromanipulators is another option for isolating single cells for subsequent processing;^{38,39} however, these systems have low compatibility with microfluidic systems. The comparison of major single cell isolation methods is provided in Table I.

To enable the use of microfluidic platforms for SC-WGS of postsurgical tissue specimens collected in a standard manner, it is essential to develop protocols suitable for tissue sample processing that allows for performing SC-WGA on target single cells within the sample in a relatively convenient manner. Therefore, in this

work, we developed a snap-frozen postsurgical endometrial tissue sample processing protocol as a guideline for preparing surgically removed tissue samples for MDA-based SC-WGA in microfluidic platforms that produces >25 ng of genomic DNA. This protocol involves fast thawing of the endometrial tissue specimen, dissociation of single cells from the tissue, labeling of epithelial cells, isolating targeted single cells, and amplifying their genome in the microfluidic platform. The SC-WGA results of epithelial cells dissociated from the endometrial tissue were compared to those of lab-cultured KLE endometrial cancer cell line. We believe that the effective postsurgical tissue processing method that enables successful genome amplification of interested single cells would serve as a guideline for performing SC-WGA on various postsurgical samples including tumors using microfluidic platforms and can be applied to a wide range of applications in biomedical research and clinical diagnosis. Beyond that, it is also possible to apply this method of tissue sample processing to perform single cell RNA sequencing of various surgically collected tissue samples in microfluidic platforms.^{40,41}

MATERIALS AND METHODS

Endometrial tissue sample collection

Endometrial tissue samples were collected aseptically and in a sterile field in pathology laboratories, immediately after hysterectomy (for a benign or malignant condition) and upon diagnostic completion. Informed consent from every patient was obtained prior to surgery under the Mayo Clinic approved Institutional Review Board (IRB 12-004445). The specific research activities utilizing the tissue samples collected under IRB 12-004445 was approved under IRB 18-004457. All methods and research activities were performed in accordance with Institutional guidelines and regulations. A pathologist assistant collected tissue samples with the assistance from a member of the research team. Endometrial samples of approximately 1 cm^3 were collected with a sterile scalpel and metal tweezer and placed in sterile 10 ml Falcon tubes that were either empty or contained 1 ml of sterile Tris-EDTA (TE) buffer. The vials were immediately snap-frozen in dry ice upon collection. The samples were kept on a -80°C freezer for long-term storage. The single cell isolation work with these tissue samples was performed under another approved Mayo Clinic IRB (18-004457).

TABLE I. A comparison of microfluidic-based single cell isolation methods.

Single cell isolation method	Features	Ideal cell number	Throughput	Suitable applications
FACS	Selection based on available fluorescent spectrum	10^5 – 10^6	High	Large-scale single cell sorting
Micromanipulators	Selection based on visually discernable traits	10^3 – 10^4	Low	Analyzing single cells from a complex community of cells
Droplet microfluidics	Encapsulation based on Poisson distribution	10^3 – 10^4	High	Analysis of single cells and a low number of cells
Optical tweezers	Selection based on visually discernable traits	10^3 – 10^4	Low	Analyzing single cells from a complex community of cells

Cell preparation and dissociation

Tissue samples

The endometrial tissue samples were fast-thawed in a water bath at 37 °C for 3–5 min, cut with scissors, and placed in cell dissociation buffer. Samples were then incubated on a shaker incubator (Thermo Fisher) at 37 °C, 500 rpm for an hour. The cell dissociation buffer was composed of 10% fetal bovine serum (FBS), 750 U/ml collagenase I, and 80 U/ml DNase I in DMEM/F-12, GlutaMAX cell culture medium. The resulting liquid was then filtered (70 μm) twice and pelleted.

Cell line

KLE endometrial cancer cell line (ATCC, CRL-1622) was cultured in DMEM/F-12, GlutaMAX (Thermo Fisher) at 37 °C and harvested and pelleted at 1000 rpm for 10 min. The cell pellet was washed once using phosphate buffer saline (PBS) and pelleted again.

Cell labeling

Staining buffer was prepared in PBS with 10% FBS and 0.1% NaN₃. 2 μl of CD9 antibody (MEM-61, Alexa Fluor 488, Novusbio) or aminopeptidase N/CD13 antibody (APN/514, Alexa Fluor 405, Novusbio) was added into 198 μl staining buffer. CD9 antibody is an epithelial cell marker, while CD13 antibody is a stromal cell marker, and these markers were chosen based on the works reported by others.⁴² Pellets of KLE cells and cells dissociated from the endometrial tissue were resuspended in staining buffer with either CD9 or CD13 antibody markers and were incubated in a closed ice bucket

placed in a 4 °C refrigerator overnight. Meanwhile, two mouse IgG1 isotype controls (Alexa Fluor 488, Alexa Fluor 405, Novusbio) were used to differentiate nonspecific background signals. These samples were then pelleted and washed once using staining buffer and resuspended in PBS with 0.02% Pluronic F127 (Sigma).

Microfluidic experimental setup

The study was performed in our optofluidic platform⁹ at Mayo Clinic (Rochester, MN). Briefly, this platform integrates a microscope (Nikon Eclipse), optical tweezers (Thorlabs), and a polydimethylsiloxane (PDMS) microfluidic chip with 12 parallel reaction systems [Fig. 1(a)]. The microvalves in the devices allow for the on-demand creation of microchambers for single cell isolation and subsequent chemical reactions [Fig. 1(b)]. The microfluidic channels for sample introduction were primed in PBS with 0.04% Pluronic F127 (Sigma) for 30 min prior to cell introduction to alleviate the problem of cells sticking to the channel surface. Briefly, the fluorescently labeled cell suspension was flowed into the sample channel. Single cells that are CD9-positive identified by the green fluorescent protein (GFP) fluorescent signal were trapped and transported into microchambers by optical tweezers [Fig. 1(c)]. Nontarget cells that inadvertently enter the chamber can be trapped and moved out of the chambers before closing the chamber gates for cell isolation. The sample channel was then flushed with PBS to wash redundant cells and substances out of the chip, and all valves are closed. Then, reagents were added into the chambers in a sequential manner. Specifically, the sample channel was flushed by the lysis buffer to remove the prior solution in the chamber. Then, the first

FIG. 1. Optofluidic platform overview. (a) An optofluidic platform consists of a microscope, laser tweezers, and a microfluidic device for bacterial SC-WGA. (b) Valve functions of the microfluidic device, capable of creating microchamber when pressurizing the control line. (c) Time-elapsd images of transporting a single cell into a chamber using an optical tweezer. The cell was dissociated from endometrial tissue labeled with CD9 antibody is outside of the chamber, and its GFP fluorescence was verified before being trapped and moved into the chamber using optical traps. (d) The schematic diagram of adding fluids into the microfluidic chambers sequentially.

gate was opened to allow for the lysis buffer to fill the first chamber. The neutralization buffer and DNA polymerase were added into the chambers in the same manner [Fig. 1(d)]. The amplified products were collected from the outlet ports of the microfluidic chip and transferred into 0.2 ml PCR tubes. All the supplies and reagents were filtered (0.2 μ m), autoclaved, or UV-sterilized, except for the DNA polymerase. Ten single cell reactions and two negative control reactions were performed in each test in a parallel fashion for 16 h. The microfluidic devices can be designed to accommodate the SC-WGA of a larger number of cells in parallel (50–60 cells/device).

Epithelial SC-WGA workflow

The general workflow is shown in Fig. 2. Dissociated and labeled cells were introduced into the microfluidic chip, and the CD9-positive cells were moved into the chambers for isolation using laser tweezers. Lysis buffer was introduced, and the microfluidic chip was incubated on a hotplate at 37 °C for 10 min, followed by the addition of the neutralized buffer to terminate DNA denaturation at room temperature. The polymerase was mixed according to the kit's instruction (Qiagen REPLI-g Single Cell Kit) and added into the reaction chambers, and the chip was placed on a hotplate at 32 °C for 16 h. The kit's manufacturer suggested an incubation at 30 °C for 8 h in standard PCR tubes; however, in this study, the incubation parameters were adapted for optimal incubation in microfluidic devices for increased yield based on earlier publications.^{9,20} The amplification reaction was terminated by incubating the microfluidic chip at 65 °C for 3 min and cooled on ice. Gel-loading pipette tips were inserted into the outlet ports of the chip, and nuclease-free water was introduced into the chip to flush the amplified product into the pipette tips until the fluid level reached the 20 μ l mark. The product was collected and stored at 4 °C, and high-sensitivity Qubit assay (Thermo Fisher) was performed to assess the amount of the amplified genomic DNA from each single cell. If the amplified genomic DNA from a single cell was >25 ng with no detectable double-stranded DNA in high-sensitivity Qubit assay, we considered it a successful amplification.

RESULTS AND DISCUSSION

Sample thawing and cell dissociation

We attempted three thawing methods prior to cell dissociation to find out the thawing condition most effective for preserving cell integrity as the liquid crystallization would be a concern for cell wall disruption. Samples were taken out of the –80 °C storage and (1) fast-thawed at 37 °C in water bath; (2) slow-thawed at –20 °C for an hour and at 4 °C for an hour; or (3) thawed at room temperature for 3 h. We also tested tissue thawing in 10% buffered formalin and without buffer along with each of the three thawing methods. Meanwhile, we also tested the impact of the different incubation times in the cell dissociation buffer. Samples that underwent overnight and 2 h of shaking incubation at 400 rpm led to no observable single cells, with only cellular debris observable. This may suggest that the shaking may be too aggressive and the sample was over-dissociated. When the incubation time was reduced to 1.5 h, very few intact cells were observed, and an hour of incubation

FIG. 2. An overview of the workflow of processing snap-frozen postsurgical uterus tissues to obtain single epithelial cells for lysis and DNA amplification in a microfluidic chip.

led to a number of intact single cells dissociated and, thus, could be managed in the microfluidic device.

Based on the number of recovered intact single cells, fast thawing followed by an hour of dissociation appeared to be the most effective (~5 single cells/100 nl) for preserving cell integrity and was chosen as the thawing approach for these samples. Fast thawing in 10% buffered formalin followed by an hour of dissociation led to the most efficient (~10 single cells/100 nl) dissociation of intact single cells from the samples (it is an agent widely used to preserve cell integrity); however, no DNA amplification was observed as formalin inhibits MDA-based amplification. The other two thawing methods (slow thawing at –20 °C, 4 °C, room temperature) did not lead to observable intact single cells after dissociation. Therefore, we continued the protocol development with the fast-thawing method (37 °C) and without added buffer. Fast thawing followed by 1.5 h of dissociation led to very few intact cells after dissociation (~1 cells/5 μ l), which is difficult to manage in microfluidic chips as the channels and chambers handle only nanoliters of volume. For the human tissues, ~10 cells/100 nl

TABLE II. Summary of experimental conditions for obtaining single cells from human endometrial tissues for single cell whole genome amplification (SC-WGA).

	Fast thaw		Slow thaw		Thaw at room temperature	
Dissociation time	No formalin	10% formalin	No formalin	10% formalin	No formalin	10% formalin
Overnight	No intact cells	No intact cells	No intact cells	No intact cells	No intact cells	No intact cells
2 h	No intact cells	No intact cells	No intact cells	No intact cells	No intact cells	No intact cells
1.5 h	Few cells (~1 cell/5 μ l)	Few cells (~1 cell/5 μ l)	No intact cells	No intact cells	No intact cells	No intact cells
1 h	Cells dissociated and amplifiable (5 cell/100 nl)	Cells dissociated but not amplifiable (10 cell/100 nl)	No intact cells	No intact cells	No intact cells	No intact cells

were observed for samples that were fast-thawed in 10% buffered formalin followed by 1 h of dissociation, and ~5 cells/100 nl were observed for samples that underwent the same condition without formalin. Experimental conditions for thawing and dissociating human endometrial tissues and their effectiveness are summarized in Table II.

We estimate that endometrial cells obtained from the snap-frozen patient endometrial tissues (1 cm³) to be 5000–10 000, well below the typical requirement for FACS procedure (10⁵–10⁶ cells in 0.3–1 ml).^{43,44} Based on previous publications on single cell isolation using optical tweezers in microfluidic devices, it is ideal to have the concentration of 1 cell/nl (10⁶ cells/ml) for easy single cell manipulation in microfluidic devices.^{9,20} Lower concentrations such as 1 cell/10 nl (10⁵ cells/ml) are workable but would require increased time to identify single cells within the channel. Concentration as low as 1 cell/100 nl (10⁴ cells/ml) could lead to difficulties or failures in finding any cells within the channels. However, cell enrichment strategies could be used to process samples with low abundance of cells. For example, it is possible to concentrate 10³–10⁴ cells into only 10 μ l by centrifugation (10⁵–10⁶ cells/ml), making sample concentration workable for SC-WGA in microfluidic devices. The number of cells required for SC-WGA varies based on the goal of different studies. In this work, we focus on developing a microfluidic-based SC-WGA suitable for processing snap-frozen surgically collected tissues.

Cell labeling

Both cultured KLE cells and cells dissociated from the endometrial tissues underwent the same cell labeling process. We labeled both KLE cell lines and patient endometrial cells with CD9 and CD13 antibody markers. Among the KLE cells, a majority of the cells appeared CD9-positive, while the rest remained nonfluorescent. In the patient samples, we observed that most of the cells were CD9-positive, only a low number of cells appeared CD13-positive, indicating a low number of endothelial cells in the patient samples tested. Moreover, the isotype controls for both CD9 and CD13 antibody markers did not show positive signals, thus validating that only cells with CD9 and CD13 surface markers would display their respective fluorescent signals. The isotype controls also help to differentiate nonspecific background signals from specific antibody signals. However, the GFP signal from the cultured KLE cells was stronger [Figs. 3(a) and 3(b)], while the cells dissociated from the endometrial tissues showed decreased signal [Figs. 3(c) and 3(d)]. This could indicate that the dissociated cells had less labeling

efficiency as some of the surface markers might have been damaged during the processing steps prior to cell labeling including collection, handling, thawing, and the dissociation process.

SC-WGA

Results show that KLE cells and cells dissociated from endometrial tissue samples reached >80% amplification success [Fig. 4(a)]. Specifically, KLE cells reached 100% amplification success with

FIG. 3. Bright field and fluorescent images of cells with fluorescent antibody markers. (a) Cultured KLE cells labeled with fluorescent markers. (b) The fluorescent image shows CD9-positive/CD13-negative cells. (c) Cell dissociated from the endometrial tissue with fluorescent markers. (d) The fluorescent image showed CD9-positive/CD13-negative cells. (e) Cell dissociated from the endometrial tissue with fluorescent markers. (f) Fluorescent image shows CD13-positive/CD9-negative cells.

FIG. 4. (a) SC-WGA results for KLE cells and epithelial cells dissociated from human endometrial tissue sample snap-frozen without buffer and in TE buffer. $N = 10$ single cells per condition, error bars represent standard deviation. (b) Estimated total reaction gain and amplification cycle for each test. (c) The TapeStation results of amplified DNA from single cells from lab-cultured KLE cell line, endometrial cells dissociated from snap-frozen tissues without buffer, and endometrial cells dissociated from tissues snap-frozen in TE buffer using the optimized dissociation protocol. The DNA was not degraded. Two single cells from each sample were shown, with a negative control of sterile PBS.

an average of 41.36 ng DNA amplified from a single cell. For the cells dissociated from tissue samples that were snap-frozen without buffer, 100% amplification was achieved, with an average of 53.73 ng of DNA amplified from a single cell. For the cells dissociated from tissue samples that were snap-frozen in TE buffer, 80% amplification was achieved with an average of 30.09 ng of DNA amplified from a single cell. Our previous work showed that it is critical that the starting DNA template is of acceptable quality to obtain >25 ng of DNA after SC-WGA in the microfluidic device. Single cell DNA treated with chemicals including enzymes and alkaline agents under certain conditions would largely decrease the amplification gain, leading to only 0–3 ng of DNA after 16 h of amplification.⁹ Our previous sequencing results (unpublished) from >25 ng DNA samples obtained from on-chip SC-WGA confirmed the quality of the amplified DNA. Thus, the possibilities of obtaining a larger amount of DNA after the amplification suggested that the template DNA released from the single cells was in acceptable integrity. Sterile PBS was used as a negative control for the on-chip amplification. None of the negative controls showed amplification, indicating clean reactions in the microfluidic device. We assessed the integrity of the amplified DNA from single cells in TapeStation (Agilent 2200, Genomic DNA Screentape, Santa Clara, CA, USA), and the results show that the DNA was not degraded [Fig. 4(c)].

Despite the fact that snap-freezing samples with or without TE buffer may lead to differences in the final amplified DNA, both reached >25 ng DNA from a single cell, sufficient for standard downstream library preparation (e.g., NEBNext Ultra 2 FS DNA Library Prep, 100 pg–500 ng input DNA) and sequencing. Additional rounds of DNA amplification can be performed to generate a larger amount of DNA to accommodate various library preparation requirements.

The total DNA amount in a single epithelial cell is estimated to be 6–30 pg;^{45,46} therefore, the total reaction gain of the SC-WGA of the tested samples in the microfluidic device is estimated to be 3000–5400 [Fig. 4(b)]. We adopted a model developed by Bourcy *et al.* to estimate the replication cycles and combined error rates of the amplification.²⁶ Briefly, the replication cycle is in logarithmic relation with total reaction gain [replication cycle $N = \log_2(G)$]. This gives an estimate of 11–12 replication cycles in our SC-WGA process. In DNA amplification, the combined error rate increases linearly with the number of reaction cycles (error rate = $\epsilon \times N/2$, where ϵ is the per-base per-cycle error rate of replication). In MDA-based reaction, the per-base per-cycle error rate of replication is reported to be $\sim 3.2 \times 10^{-6}$. Therefore, we estimate that the total error rate in the amplification of the tested samples to be in the range of 1.6×10^{-5} – 1.8×10^{-5} , in agreement with previously reported combined error rate resulting from MDA-based reactions.^{26,47} In the future, we plan to use this approach to perform SC-WGA and sequencing on endometrial cells from endometrial cancer patients and analyze the relevance of microbial colonization involved in the disease. We will also experimentally characterize the genome recovery, amplification bias, and contamination profile resulting from this single cell dissociation method. This protocol can also be adapted to handle other clinical tissue samples collected in a manner standard for genomic studies without relying on the use of procedures standard for FACS-based applications to obtain single cells for SC-WGA.

CONCLUSIONS

Single cell whole genome sequencing has found various applications in lab-cultured species using microfluidic technologies. However, it has rarely been applied to clinical samples, and one of the major hurdles is the complex nature of these samples and, thus, requirements for additional sample processing steps prior to standard microfluidic experimentation procedures. Besides, due to logistic challenges of processing these samples instantly after collection, most of the samples require proper ways of storage that preserve the cell integrity without using amplification-inhibitive agents. This work focused on developing sample processing methods suitable for performing microfluidic-based single cell whole genome amplification on epithelial cells in endometrial tissues removed surgically. The method includes cell dissociation, cell labeling, and on-chip amplification. 80%–100% of single cells were amplified to >25 ng genomic DNA, sufficient for downstream processing. This work provides a guideline for processing postsurgical tissue specimens to obtain intact single cells of interest for performing SC-WGA in microfluidic devices and can potentially be adapted to other types of samples removed surgically such as tumors. Ultimately, we envision that it would be possible to perform single cell genomic studies on a vast range of clinical biospecimens in various research and diagnostic settings using microfluidic platforms.

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