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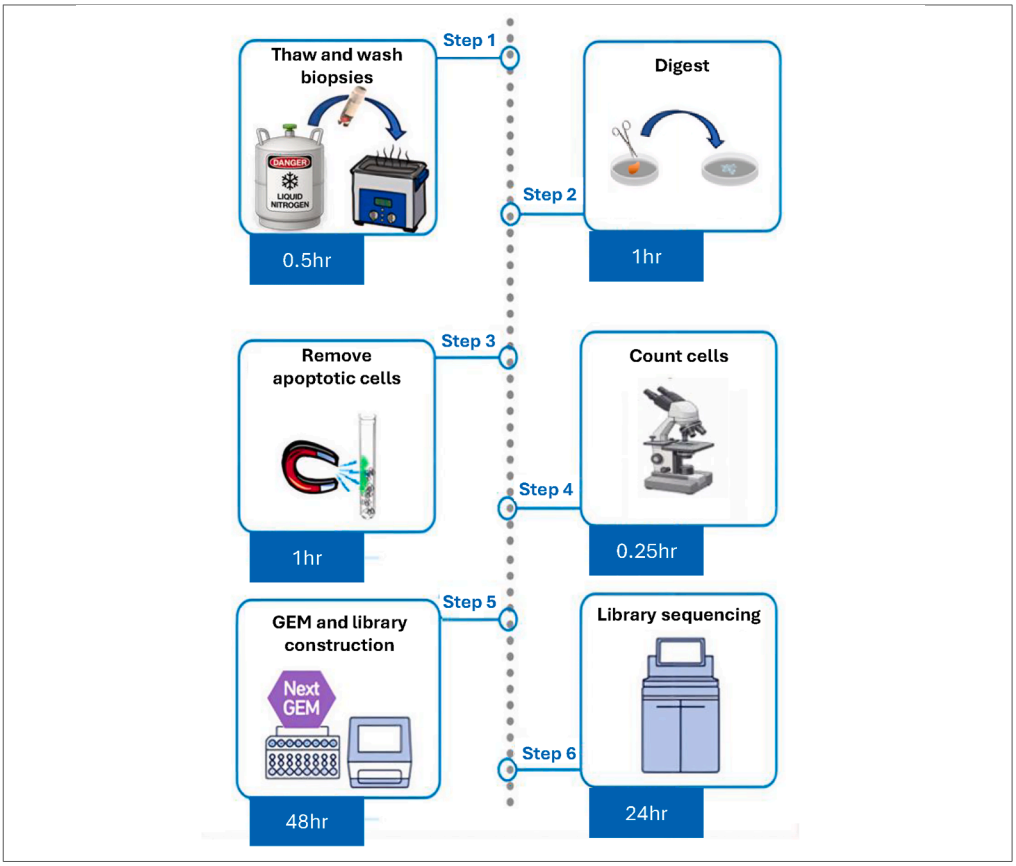
Protocol for isolating cells from a single cryopreserved gastrointestinal endoscopic biopsy for single-cell RNA sequencing



Techniques exist to isolate cells from fresh intestinal tissue; however, approaches for recovering viable cells from cryopreserved duodenal and colonic specimens are lacking. Here, we present a protocol for isolating cells from a single cryopreserved gastrointestinal endoscopic biopsy for single-cell RNA sequencing (scRNA-seq). We describe steps for thawing and washing cryopreserved endoscopic biopsies, enzymatic digestion of biopsies, and purification preparation. We then detail procedures for dead cell removal, quantification of cell recovery, and 10X GEM generation and library preparation.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Guidance on cellular extraction from a single cryopreserved gastrointestinal endoscopic biopsy

Isolation of defined immune and non-immune subsets from clinically relevant material

Procedures for single-cell characterization and immune profiling

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Protocol for isolating cells from a single cryopreserved gastrointestinal endoscopic biopsy for single-cell RNA sequencing

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SUMMARY

Techniques exist to isolate cells from fresh intestinal tissue; however, approaches for recovering viable cells from cryopreserved duodenal and colonic specimens are lacking. Here, we present a protocol for isolating cells from a single cryopreserved gastrointestinal endoscopic biopsy for single-cell RNA sequencing (scRNA-seq). We describe steps for thawing and washing cryopreserved endoscopic biopsies, enzymatic digestion of biopsies, and purification preparation. We then detail procedures for dead cell removal, quantification of cell recovery, and 10X Gel Bead-in-Emulsion (GEM) generation and library preparation.

For complete details on the use and execution of this protocol, please refer to Richards et al.[1]

BEFORE YOU BEGIN

scRNA-seq is an integral tool for profiling and characterizing the cellular composition of peripheral blood samples but can also be applied to tissue specimens with appropriate dissociation methods to yield single-cell suspensions. Detailed immune profiling and scRNA-seq characterization of Ulcerative Colitis[2] and Crohn's Disease[3] endoscopic biopsies have highlighted the roles of various immune and non-immune cellular subsets, and these approaches have enabled new target identification and patient stratification approaches for various therapeutics. Seminal studies using endoscopic biopsies have required the utilization of multiple pooled endoscopic biopsies[4] or full tissue resections[5] to efficiently liberate and perform detailed immune phenotyping. Although useful in the context of academic research,[6] scRNA-seq profiling in the clinical setting has been challenging[7] due to the limited nature of patient tissue. Moreover, the integration of scRNA-seq into clinical trials has been limited due to constraints on tissue access and the need for cryopreservation. Therefore, developing protocols to enable detailed tissue characterization from a single cryopreserved endoscopic biopsy is a priority to enable deployment of scRNA-seq in clinical studies and detailed mechanism of action studies.

Tissues with mucosal layers and viable epithelial layers, such as those lining the gastrointestinal (GI) tract prove challenging to robustly dissociate and prepare for these methods.[8] Fresher specimens are generally more conducive, and while cryopreserved samples are more

convenient for scaling protocols across clinical sites, sample handling and storage, this comes as a further detriment to maintain viable cells through dissociation. This protocol outlines the specific enzyme cocktails and processing steps to optimize cell recovery yield and viability of endoscopic biopsies collected from the duodenum, ileum or colon, and to remove dead cells to maximize scRNA-seq data quality. The procedure for preparing these cell suspensions for scRNA-seq concludes this protocol.

Innovation

Inflammation of the upper and lower gastrointestinal tract is a global disease, with increasing prevalence.[9] Despite various treatment options, inadequate response to therapeutics in patients with coeliac and inflammatory bowel disease remains a key unmet medical need. Detailed tissue characterization within these patient populations is required to enable new target identification, combination therapy strategy, and patient stratification.

To evaluate immune and non-immune cellular subsets in the context of health and disease, we established a protocol to efficiently liberate significant numbers of cells from a single cryopreserved endoscopic pinch biopsy from the duodenum and colon. This protocol outlines optimal cryopreservation, cellular dissociation, processing, and downstream scRNA-seq approaches. These studies have enabled the detailed immunophenotyping of patients using a single cryopreserved endoscopic pinch biopsy, thus enabling the interrogation of mechanisms of action and drug treatment response within a clinical trial setting.

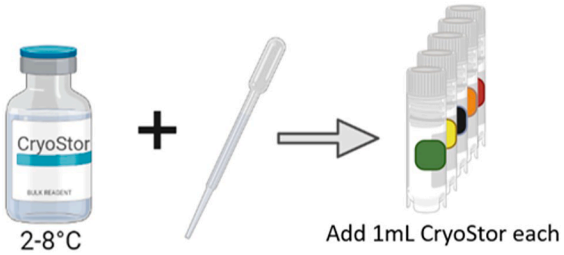
In conclusion, we describe an optimized protocol for cellular characterization and subsequent scRNA-seq of single cryopreserved endoscopic biopsies, thereby reducing patient sampling burden and resource requirements.

Institutional permissions

Endoscopic duodenal, ileal and colon biopsies are class BSL-2 biological hazards with high potential risk of contamination of work areas as well as personnel handling these samples. All work should be performed in a BSL-2 designated laboratory environment with appropriate personnel protective equipment (PPE) and engineering controls established by the institution. At a minimum, work should be performed completely within a ventilated biosafety cabinet to reduce the risk of personnel exposure. Generation of aerosols should be reduced whenever possible (i.e., pipetting and mixing, centrifugation), and all work surface areas and implements coming into contact, or potentially contacting, the samples themselves or intermediary buffers and washes must be thoroughly cleaned with institution-approved disinfectant (i.e., fresh 10% bleach solution or equivalent).

Materials for biopsy cryopreservation

Cryopreservation vials should be filled with 1mL of CryoStor™ or another cryopreservation medium. Endoscopic biopsies should be added to the cryopreservation vial and fully submerged in the CryoStor™ liquid. Cryopreservation vials with biopsies should be placed inside a cryopreservation container like CoolCell™ container at 20–25°C and stored in a −70°C/−80°C freezer overnight. If an ultracold freezer isn't available, bury the CoolCell™ in enough dry ice to last until shipment on dry ice the following day.



The day of the assay

Timing: 30 min

1. Prepare enzymatic digestion buffer.
   a. Obtain 1 vial each of frozen Liberase™ TM and DNase aliquots per four biopsies and place on a rotator until thawed (10–20 min).
   b. For each endoscopic biopsy to process, add 0.15 mL of Liberase™ TM stock and 0.075 mL of DNase stock to 3.53 mL of wash media. This volume is 1.25X the volume used for processing.
   c. For each endoscopic biopsy to be processed, add 2.75 mL of digestion buffer to a 4 mL polypropylene round-bottom tube and 0.25 mL to a microcentrifuge tube.
   d. Keep digestion buffer (both the stock preparation and aliquots allocated to round-bottom and microcentrifuge tubes) on ice.
2. Transfer 20 mL/sample of thawing media to a 50 mL tube in a 37°C water bath.
3. Transfer 25 mL of RPMI-1640 for the experimental setup and keep at 20–25°C.
4. Maintain the dead cell removal buffer at 20–25°C for the start of the experiment.

Note: If using lyophilized reagents for single use, prepare as indicated in step 1.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
PBS w/o Calcium, Magnesium	ThermoFisher	J67802.k2
Bovine Serum Albumin	Sigma	A6003

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Liberase TM	Sigma	5401127001-10MG
DNase	Sigma	D5025-15KU
Heat Inactivated Fetal Bovine Serum	ThermoFisher	16140071
$CaCl_2$	Promocell	C-34006
RPMI 1640	ThermoFisher	72400146
EasySep Dead Cell Removal Kit	Stemcell Technologies	17899
ViaStain AOPI Staining Solution	Revvity	CS2-0106-5ML
SPRIselect	Beckman	B23318
Low EDTA TE (1X), pH 8.0	Quality Biological	351-324-721
Buffer EB	Qiagen	19086
High Sensitivity D5000 Reagents containing Sample Buffer and Ladder	Agilent Technologies	5067-5593
High Sensitivity D1000 Reagents containing Sample Buffer and Ladder	Agilent Technologies	5067-5585
Illumina Library Quantification Kit ROX Low	Roche	07960336001
Tween 20	Sigma	P7949-500ML
Sodium Hydroxide, 10 N	Alfa Aesar	J63736-AE
1 M Tris HCl Buffer, pH 8.0	ThermoFisher Scientific	J22638-AP
Ethanol, Pure, 200 Proof	Decon Labs	V1001
Other		
MACSmix™ Tube Rotator	Miltenyi Biotec	Cat# 130-090-753
Straight stainless-steel surgical scissors 3-5 inches		
37 °C water bath large enough to accommodate a rack of 4 mL tubes	Thermofisher	Cat#TSGP05
37 °C incubator large enough to hold MACSmix™ Tube Rotator	Benchmark Scientific	Cat#H3565-45
For plate-based dead cell removal - EasyPlate™ EasySep™ Magnet	Stemcell Technologies	Cat# 18102
For tube-based dead cell removal - EasyEights™ EasySep™ Magnet	Stemcell Technologies	Cat# 18103
Polypropylene 15 mL centrifuge tube	ThermoFisher	Cat# 339651
Eppendorf Safe-Lock Tubes	Eppendorf	Cat# 022600044
MACS SmarStrainers 70 mm	Miltenyi	130-110-916
MACS SmarStrainers 30 mm	Miltenyi	130-110-915
Falcon® 5 mL Round Bottom High Clarity Polypropylene Test Tube, with Snap Cap	Corning	Cat# 352063
Corning® Falcon® Round Bottom Test Tubes	Corning	Cat# CLS352057
Cellaca PLX low fluorescence slides	Revvity	CHM2-ACR-001
Chromium Next GEM Single Cell 3′ Kit v3.1	10X Genomics	1000268
Next GEM Chip G	10X Genomics	2000177
High Sensitivity D5000 ScreenTape	Agilent Technologies	5067-5592
High Sensitivity D1000 ScreenTape	Agilent Technologies	5067-5584
Dual Index Kit TT Set A	10X Genomics	1000215
PhiX Control V3	Illumina	FC-110-3001

MATERIALS AND EQUIPMENT

Liberase ™ TM solution

Reagent	Final concentration	Amount
Deionized water	N/A	4 mL
Liberase TM	2.5 mg/mL	10 mg

- Mix by rotating until fully dissolved (Do not vortex).
- Aliquot into cryovials at 650 µl/vials (enough for 4 samples).
- Store at −20 °C and use within 7 days.

DNase solution		
Reagent	Final concentration	Amount
0.9% Saline	N/A	1.5 mL
DNase	5 mg/mL	7.5 mg

- Mix by rotating until fully dissolved (Do not vortex).
- Aliquot into cryovials at 300 μl/vial (enough for 4 samples).
- Store at −20°C and use within 7 days.

Note: If working with more than four endoscopic specimens, lyophilized Liberase ™ TM and DNase can be prepared the day of assay for single use.

Thawing medium		
Reagent	Final concentration	Amount
RPMI 1640	N/A	500 mL
Heat Inactivated fetal bovine serum (HI FBS)	10%	50 mL

- Mix by inverting bottle 10X.
- Sterile filter using 0.22 μM bottle top filter.
- Store at 4°C and use within 7 days.

Wash Media		
Reagent	Final concentration	Amount
PBS without Ca^{2+}, Mg^{2+}	N/A	500 mL
BSA	1%	5 g

- Mix using stir bar until completely dissolved.
- Sterile filter using 0.22 μM bottle top filter.
- Store at 4 °C and use within 7 days.

Digestion buffer (per sample)		
Reagent	Final concentration	Amount
Wash media	N/A	3.53 mL
Liberase solution	4 %	0.15 mL
DNase solution	2%	0.075 mL

- Thaw and slowly mix on a rotator the vials of DNase and Liberase ™ TM.
- Each sample requires 3 mL of digestion buffer.
  - 0.075 mL of DNase, 0.15 mL of Liberase ™ TM and 3.53 mL wash media = 3.75 mL (1.25X extra).
- For each sample, add 2.75 mL to a 4 mL polypropylene tube and 0.250 mL to an Eppendorf tube.
- Store at 4°C and use within 7 days.

Dead cell removal buffer		
Reagent	Final concentration	Amount
PBS without Ca^{2+}, Mg^{2+}	N/A	1000 mL
HI FBS	2%	20 mL
$CaCl_2$	1 mM	1 mL

- Mix by inverting the bottle 10X.
- Sterile filter using a 0.22 μM bottle top filter.
- Store at 4°C until use within 7 days.

STEP-BY-STEP METHOD DETAILS

Thawing and washing cryopreserved endoscopic biopsies

Timing: ~0.5 h for four endoscopic biopsies

In this step, we outline the procedure to quickly thaw cryopreserved endoscopic biopsies stored at liquid nitrogen or −80°C. Step 3 onward must be performed in a biological safety cabinet with appropriate ventilation and PPE. Note that vials must be kept in dry ice for transit from the liquid nitrogen or freezer before thawing and during the first step below. Ensure a water bath is heated to 37°C before removing samples from frozen storage.

1. Wrap each cryopreserved biopsy vial in parafilm where the cap touches the tube to create a barrier in case of unexpected pressure release during thawing. Keep on dry ice until all samples are ready to thaw.
2. Place wrapped vials into a floating foam vial rack and submerge them just to the level of the cryopreservation media in a 37°C water bath until the ice begins to melt (1.5 to 3 min, depending on the volume of media).

   Note: Periodic shaking of the vials is recommended to facilitate even thawing.

3. Immediately transfer vials to a biosafety cabinet, and one at a time, carefully remove the parafilm, slowly open the cap with a small wipe in case of discharge, and gently pour into a 50 mL tube per sample. Gently swirl to mix and thaw any remaining ice.

   ⚠ CRITICAL: Minimize time at 20–25°C in the partially thawed state.

   ⚠ CRITICAL: Maintain the same processing order at each step as the thawed order.

   Note: For samples > 0.5 cm, manually dissect the sample into < 0.5 cm pieces and distribute each piece to its own tube.

4. Add 5 mL of pre-warmed thawing media, approximately 1 drop per sec down the inside wall of the tube, minimizing turbulence in the sample.
5. Add an additional 15 mL of thawing media, approximately 1 mL per sec down the inside wall of the tube and gently swirl to mix.
6. Gently pour the biopsies over a 70 mm strainer atop a new 50 mL tube.
7. Wash biopsies with 25 mL of room-temperature RPMI-1640 (no FBS) in 5 mL increments, washing each sample with one increment before repeating.

8. Wash the biopsies with 5 mL of wash buffer.

Enzymatic digestion of biopsies and purification preparation

Timing: ~1.5 h for four endoscopic biopsies

In this step, we describe the process for enzymatically digesting thawed biopsies and preparing them for the removal of dead and dying cells. Apart from steps 12 and 13 (incubating the samples in the digestion buffer at 37°C) and 18 and 22 (centrifugation), all steps must be performed in a biological safety cabinet with appropriate ventilation and PPE. Maintain the processing order the same as the thaw order.

9. Add each biopsy to individually prepared microcentrifuge tubes containing 0.25 mL of digestion buffer and carefully mince with 4.5-inch surgical scissors until no pieces are visible.
10. Transfer suspensions to the prepared round-bottom tubes containing 3 mL of digestion buffer and rinse the microcentrifuge tube with 1 mL from the round-bottom tube twice to collect as much of the suspension as possible.
11. Fully close the cap and seal it by wrapping it in parafilm.
12. Place tubes into the 37°C water bath for 5 min.
13. Transfer the tubes to a rotator and set it to gently rotate end-over-end for 30 min in a 37°C incubator.
    a. If using a MACSmix rotator, use the top setting (thick line).
14. After incubation, carefully remove the parafilm and add 60 μL of straight FBS directly to each tube. Resnap the caps to seal and mix by gently inverting the tubes twice.
15. Pour the contents of each tube over a 70 mm strainer nested over a 30 mm strainer (remove the bottom half of the 70 mm strainer to fit atop a 15 mL conical polypropylene centrifuge tube).
16. Wash the round-bottom digestion tubes with 4 mL of dead-cell removal buffer and pour the rinses over their respective sample strainers. Wash the strainers twice with 3 mL of dead cell removal buffer.
17. Close each tube and mix by gently inverting five times.
18. Centrifuge samples at 300 *g* for 10 min at 20–25°C in a swinging rotor centrifuge.

Note: If available, rotor bucket sealed lids should be used during all centrifugation steps to minimize the risk of aerosols and contamination of the instrument should a tube be damaged.

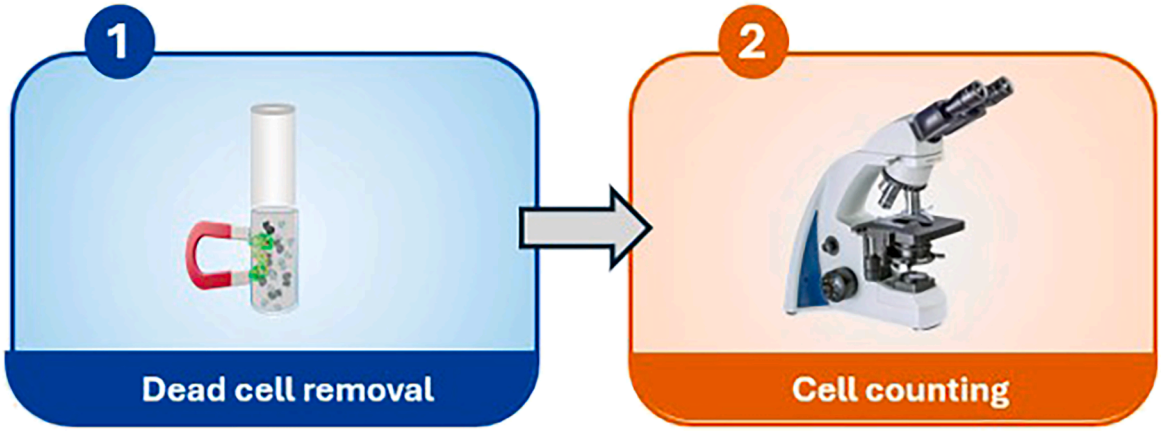
19. Return the tubes to the biological safety cabinet and remove supernatants by gently decanting them to a waste receptacle.

Note: Depending on the size and composition of the starting material, visible pellets may be unlikely. Translucent cell smears may be visible at the bottom of the tube, but lack of a discernible pellet is NOT cause for alarm.

20. Resuspend the cells in the residual buffer by gently tapping the tubes.
21. Add 4 mL of dead cell removal buffer to each sample.
22. Centrifuge samples again at 300 *g* for 10 min at 20–25°C.
23. Return the tubes to the biological safety cabinet and carefully remove as much supernatant as possible using an aspirating pipet.
24. Resuspend each sample in 100 μL of dead cell removal buffer, then gently mix with a wide-bore pipette tip.
25. Let tubes rest for 3 min at 20–25°C before proceeding to the next step.

Dead cell removal and quantification of cell recovery

Timing: ~1.5 h for four endoscopic biopsies



In this step, we conclude biopsy processing by purifying dead and dying cells to maximize viable cell input for scRNA-seq. These data are collected as a final step using cell-counting methods. Note that the protocol to this point is equally applicable to flow cytometry, where the measurement of dead and dying cells can be incorporated into the panel. As in the prior two sections, all steps except centrifugation and counting must be performed in a biological safety cabinet with appropriate ventilation and PPE. Before beginning, set the centrifuge to 4°C.

Note: One of two protocols may be used to remove dead/dying cells. A plate-based method is faster; however, there may be some cell loss. A tube-based method is slower but results in less cell loss.

Plate method

a. Transfer each sample to a different well of a 96-well polystyrene round-bottom plate.
b. Add 5 μL of Dead Cell Removal Cocktail directly to each sample.

⚠ CRITICAL: DO NOT vortex the cocktail tube! Mix by gently tapping or inverting.

c. Gently swirl the plate to mix.
d. Add 5 μL of Biotin Cocktail directly to each sample.

⚠ CRITICAL: DO NOT vortex the biotin tube! Mix by gently tapping or inverting.

e. Gently swirl the plate to mix.
f. Incubate the plate at 20–25°C for 3 min.

Note: During incubation, vortex the RapidSpheres tube at high speed for 30 s.

g. Add 10 μL of RapidSpheres directly to each sample, then mix each sample with a fresh wide-bore tip.
h. Immediately add 150 μL of dead cell removal buffer and place the plate on an EasySep plate magnet.
i. Incubate the plate at 20–25°C for 10 min.
    i. During incubation, add 5 mL of of cold final wash buffer (pre-cooled to 4°C) to fresh 15 mL tubes (one tube per sample).
j. For each sample, with the plate still on the magnet, transfer the buffer containing viable cells to the 15 mL tube containing 5 mL of cold final wash buffer carefully without disturbing the beads.
k. Centrifuge samples at 300 *g* for 5 min at 4°C.
l. Return the tubes to the biological safety cabinet, remove the supernatants by gently decanting into a waste receptacle, then resuspend the residual buffer by gently tapping each tube.
m. Add 5 mL of cold final wash buffer to each sample and gently invert to mix.
n. Centrifuge samples again at 300 *g* for 5 min at 4°C.
o. Return the tubes to the biological safety cabinet and carefully decant to a waste receptacle.
p. Pulse vortex at low speed and let sit on ice for at least 3 min before counting.
q. Count cells using acridine orange and propidium iodide with a compatible cell counter.

Tube method

a. Add 5 μL Dead Cell Removal Cocktail to the tubes containing dissociated cells.

⚠ CRITICAL: DO NOT vortex tubes! Gently swirl the tube to mix.

b. Add 5 μL of Biotin Cocktail.

⚠ CRITICAL: DO NOT vortex tubes! Gently swirl the tube to mix.

c. Incubate at 20–25°C for 3 min.

Note: During incubation, vortex the RapidSpheres tube at high speed for 30 s.

d. Add 10 μL of RapidSphere and mix by swirling the tube.
e. Immediately add 2.5 mL of dead cell removal buffer, then place the tube on an EasySep tube magnet.
f. Incubate for 10 min at 20–25°C.
    i. During incubation, add 2.5 mL of 4°C final wash buffer to fresh 15 mL tubes (one tube per sample).
g. While the tube remains on the magnet, remove the buffer containing viable cells and carefully transfer it to the 15 mL tube containing 2.5 mL of cold final wash buffer, without disturbing the beads.
h. Centrifuge samples at 300 *g* for 5 min at 4°C.
i. Return the tubes to the biological safety cabinet, remove the supernatants by gently decanting into a waste receptacle, then resuspend the residual buffer by gently tapping each tube.
j. Add 5 mL of 4°C cold final wash buffer to each sample and gently invert to mix.
k. Centrifuge samples again at 300 *g* for 5 min at 4°C.
l. Return the tubes to the biological safety cabinet and carefully decant to a waste receptacle.
m. Pulse vortex at low speed and let the tubes sit on ice for at least 3 min before counting.
n. Count cells using acridine orange and propidium iodide with a compatible cell counter.

10X GEM generation and library preparation

Timing: ~8 h for four endoscopic biopsies

26. Prepare the GEM master mix from the Next GEM Single Cell 3′ kit by combining 18.8 μL of RT Reagent B, 2.4 μL Template Switch Oligo, 2.0 μL Reducing Agent B, and 8.7 μL RT Enzyme C per sample. Mix gently 15 times with a pipette, then briefly centrifuge to bring down.
27. Transfer 31.9 μL of master mix to PCR tubes and keep on ice.
28. Assemble the Chromium Next GEM chip per the manufacturer's instructions.
29. Load a volume of cell suspension per sample to yield 16,500 cells, making up the difference with water to a maximum of 44 μL volume per PCR tube.
30. Gently mix the master mix with the cell suspension, then transfer 70 μL to row 1 of the GEM chip.
31. Vortex the Gel Bead strip for 30 sec.
32. Briefly centrifuge the Gel Bead strip for 5 sec and confirm there are no bubbles at the bottom of the tubes.
33. Slowly dispense 50 μL of Gel Beads to row 2 of the GEM chip, wait 30 sec before proceeding.
34. Dispense 45 μL of Partitioning Oil to row 3 of the GEM chip and close the lid.
35. Process the CHIP on the Chromium Controller (approximately 18 min) and visually inspect for clogs.
36. Slowly aspirate 100 μL of GEMs from row 3 and dispense them over 20 sec into a new PCR tube on ice. Do not let the tube sit on ice for longer than 1 hr.
37. Incubate on a thermocycler under the following parameters:
    a. Lid temperature = 53 °C, Reaction volume = 125 μL, Run time = 55 min
    b. Step 1: 45 min at 53°C
    c. Step 2: 5 min at 85°C
    d. Step 3: Hold at 4°C

Note: Samples can be stored at 4 °C for 72 hr, −20 °C for one week, or used immediately

38. Thaw the Cleanup Buffer at 65°C for 10 min in a thermomixer. Cool to at 20–25°C.
39. Add 125 μL of Recovery Agent to each sample at 20–25°C and wait 2 min. DO NOT pipette or vortex to mix.
40. Slowly remove and discard 125 μL of Recovery Agent + Partitioning Oil (pink) from the bottom of the tube. DO NOT aspirate the aqueous (clear) sample at the top.
41. Vortex Dynabeads > 30 sec before using.
42. Prepare Dynabeads Cleanup mixby combining 182 μL Cleanup Buffer, 8 μL Dynabeads, 5 μL Reducing Agent B, and 5 μL nuclease free water per sample.
43. Vortex the cleanup mix thoroughly and add 200 μL to each sample, pipetting ten times to mix.
44. Incubate for 10 min at 20–25°C with the caps OPEN, mixing again by pipetting after 5 mins of incubation.
45. Prepare Elution Solution I by combining 98 μL Buffer EB, 1 μL 10% Tween 20, and 1 μL Reducing Agent B per sample. Vortex to mix and briefly centrifuge to bring the contents of the tube to the bottom.
46. After the 10 min incubation, place the tubes on a magnetic separator until the samples clear, and then remove and discard the supernatant containing the aqueous phase and Recovery agent.
47. Add 300 μL of 80% ethanol to the pellet and wait 30 sec.
48. Remove the ethanol and add an additional 200 μL of 80% of the ethanol to the pellet, waiting an additional 30 sec.
49. Remove the ethanol and centrifuge the tubes briefly.
50. Place tubes on the magnetic stand, remove residual ethanol, and allow the tubes to air dry for 1 min.
51. Remove the tubes from the magnet and immediately add 35.5 μL Elution Solution I. Mixwith a pipette without forming bubbles.
52. Incubate 2 min at 20–25°C and return the tubes to the magnet until samples clear.

53. Transfer 35 μL of each sample to a new PCR tube.
54. Prepare cDNA Amplification Mix on ice by combining 50 μL Amp Mix and 15 μL cDNA primers per sample. Vortex gently to mix and briefly centrifuge to bring the contents of the tube to the bottom.
55. Add 65 μL cDNA Amplification Mix to each 35 μL of purified sample, pipetting fifteen times to mix. Briefly centrifuge to bring down.
56. Incubate on a thermocycler under the following parameters:
    a. Lid temperature = 105 °C, Reaction volume = 100 μL, Run time = 30-45 min
    b. Step 1: 3 min at 98°C
    c. Step 2: 15 sec at 98°C
    d. Step 3: 20 sec at 63°C
    e. Step 4: 1 min at 72°C
    f. Repeat steps 2–4 for 11–13 cycles, depending on targeted cell recovery (11 cycles: >6000 cells, 12 cycles: 500–6000 cells, 13 cycles: <500 cells)
    g. Step 5: 1 min at 72°C
    h. Step 6: Hold at 4°C

Note: Samples can be stored at 4°C for 72 h, −20°C for one week, or used immediately

57. Vortex the SPRIselect reagent to resuspend and add 60 μL to each sample and pipette fifteen times to mix.
58. Incubate for 5 min at 20–25°C, then place on the magnet until the solution clears.
59. Remove the supernatant and add 200 μL of 80% ethanol; incubate for 30 sec.
60. Remove the ethanol and perform two more washes with 80% ethanol.
61. Centrifuge the tubes briefly, place the tubes back on the magnet, remove residual ethanol, and allow to air dry for 1 min.
62. Remove the tubes from the magnet, add 40.5 μL Buffer EB, mix by pipetting 15 times, and incubate for 2 min at 20–25°C.
63. Return the tubes to the magnet, let stand until the solution clears, and transfer 40 μL to a new PCR tube.
64. Samples can be stored at 4°C for 72 hr, −20°C for four weeks, or used immediately.
65. QC the amplified cDNA samples by using the Agilent TapeStation
    a. Analyze 2 μL of each sample using the Agilent D5000 High Sensitivity ScreenTape to obtain the cDNA concentration. Calculate the cDNA yield for each sample using the cDNA concentration obtained from the Agilent TapeStation and the total volume of each sample
    b. See Figure 2 in expected outcomes
66. Vortex the Fragmentation Buffer, verify there is no precipitate, and combine 5 μL with 10 μL of Fragmentation Enzyme per sample. Store on ice.
67. Transfer 10 μL of each purified cDNA sample to new PCR tubes, then add 25 μL Buffer EB.
68. On ice, add 15 μL Fragmentation mix to each sample. Pipette fifteen times to mix and briefly centrifuge to bring the contents of the tube to the bottom.
69. Incubate on a thermocycler pre-chilled to 4°C under the following parameters:
    a. Lid temperature = 65°C, Reaction volume = 50 μL, Run time = 35 min
    b. Step 0: Pre-cooled to 4°C
    c. Step 1: 5 min at 32°C
    d. Step 2: 30 min at 65°C
    e. Step 3: Hold at 4°C
70. Vortex the SPRIselect reagent to resuspend, then add 30 μL to each sample and pipette 15 times to mix.
71. Incubate for 5 min at 20–25°C, then place on the magnet until the solution clears.
72. Transfer 75 μL of each supernatant to a new PCR tube.

⚠ CRITICAL: DO NOT discard the supernatant.

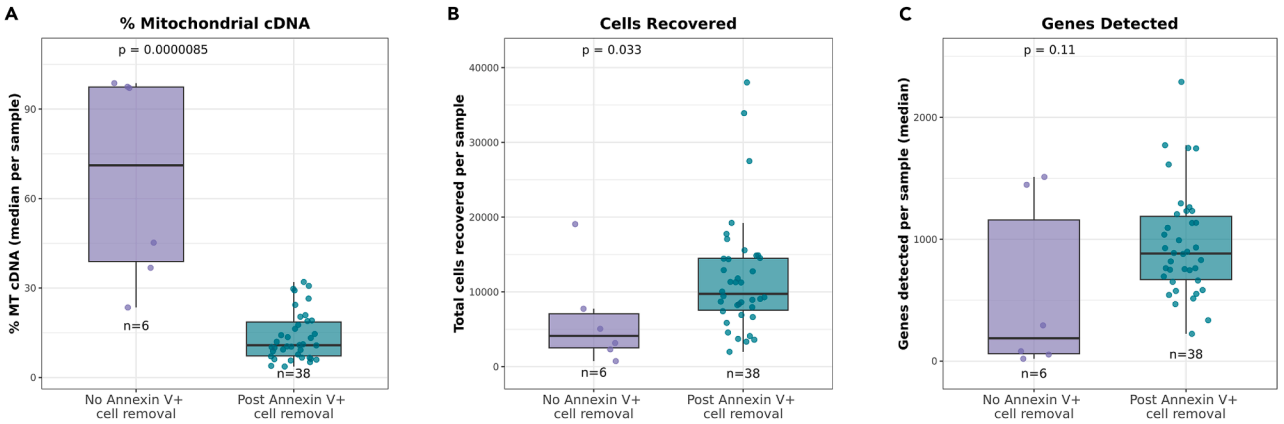


Figure 1. Annexin V+ cell depletion is associated with improved scRNA-seq quality metrics in cryopreserved endoscopic biopsies

Samples processed without Annexin V+ cell removal (n = 6) were compared to samples processed after Annexin V+ cell removal (n = 38). Metrics were calculated prior to cell-level quality control filtering. Each point represents one biological sample. P values were calculated using a two-sided Wilcoxon rank-sum test.

(A) Percentage of mitochondrial-derived transcripts (median per sample): 66.4 ± 35.0% (range 23.5–98.7%) without Annexin V+ cell removal and 13.7 ± 8.0% (range 3.7–32.0%) after Annexin V+ cell removal ($p = 8.5 \times 10^{-6}$).

(B) Total cells recovered per sample: 6,346 ± 6,680 (range 738–19,061) without Annexin V+ cell removal and 11,843 ± 7,719 (range 1,986–38,013) after Annexin V+ cell removal (p = 0.033).

(C) Median genes detected per sample: 568 ± 713 (range 19–1,512) without Annexin V+ cell removal and 961 ± 439 (range 224–2,291) after Annexin V+ cell removal (p = 0.11).

73. Vortex the SPRIselect reagent to resuspend and add 10 µL to each supernatantand pipette fifteen times to mix.
74. Incubate for 5 min at 20–25°C and then place on the magnet until the solution clears.
75. Remove and discard 80 µL of supernatant.
76. Add 125 µL 80% ethanol to the pellet and incubate 30 sec.
77. Remove the ethanol and repeat the wash with 80% ethanol.
78. Centrifuge the tubes briefly, then place them back on the magnet and remove the residual ethanol. Air dry for 1 min.
    a. Avoid over-drying to maximize elution.
79. Remove samples from the magnet and add 50.5 µL Buffer EB to each sample, mixing by pipette fifteen times.
80. Incubate for 2 min at 20–25°C, then return the samples to the magnet until the solution clears.
81. Transfer 50 µL of each sample to new PCR tubes.
82. Prepare the Adaptor Ligation Mix by combining 20 µL of Ligation Buffer, 10 µL DNA Ligase, and 20 µL Adaptor Oligos per sample. Mix by pipetting and briefly centrifuge to bring the contents of the tube to the bottom.
83. Add 50 µL of Adaptor Ligation Mix to each sample and pipette fifteen times to mix. Centrifuge briefly to bring the contents of the tubes to the bottom.
84. Incubate on a thermocycler under the following parameters:
    a. Lid temperature = 30°C, Reaction volume = 100 µL, Run time = 15 min
    b. Step 1: 15 min at 20°C
    c. Step 2: Hold at 4°C
85. Vortex the SPRIselect reagent to resuspend, add 80 µL to each sample, and pipette fifteen times to mix.
86. Incubate for 5 min at 20–25°C and then place on the magnet until the solution clears.
87. Remove the supernatant, add 200 µL of 80% ethanol, and incubate 30 sec.
88. Remove the ethanol and repeat the wash with 80% ethanol.
89. Centrifuge the tubes briefly, place the tubes back on the magnet, and remove the residual ethanol. Air dry for 2 min.

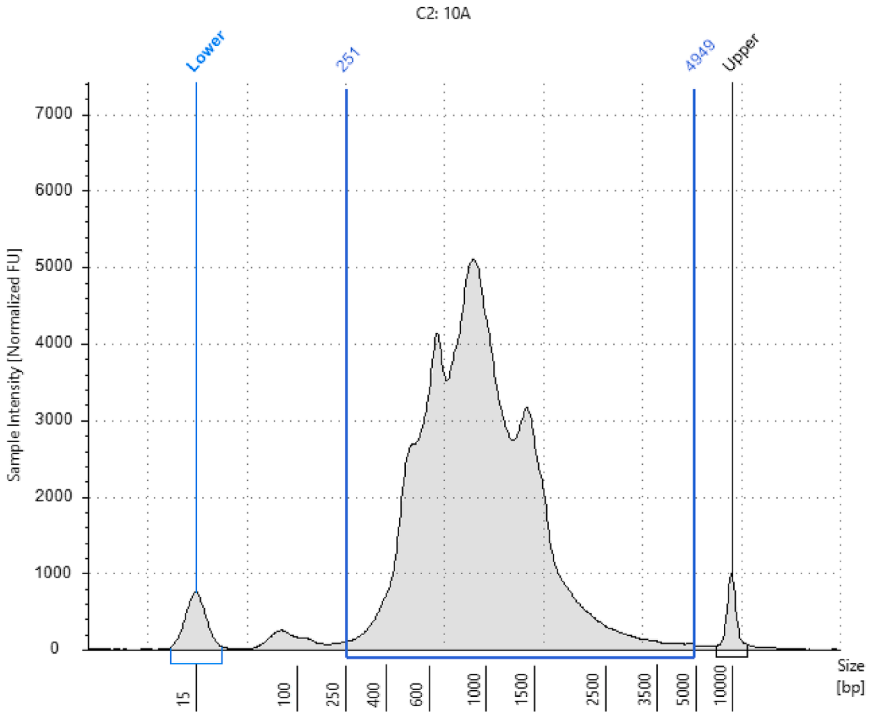


Figure 2. Representative trace from the amplified cDNA QC

The concentration (Conc. [pg/mL]) of the amplified cDNA is calculated by defining regions for each sample in the Region Settings of the TapeStation Analysis software. Regions are defined using a From [bp] and a To [bp] as shown in the graph. In the graph, the From [bp] = 251 and the To [bp] = 4949. The concentration can be obtained from the Region Table tab in the software.

a. DO NOT exceed 2 min to maximize elution.

90. Remove samples from the magnet and add 30.5 µL Buffer EB, pipetting fifteen times to mix.
91. Incubate 2 min at 20–25°C and then return samples to the magnet until the solution clears.
92. Transfer 30 µL of each sample to new PCR tubes.
93. If preparing 2 more samples, select the appropriate sample index to avoid overlap.
94. Add 50 µL of Amp Mix to each 30 µL sample.
95. Add 20 µL of appropriate Index TT. Pipette five times to mix and centrifuge briefly to bring down.
96. Incubate on a thermocycler under the following parameters:
    a. Lid temperature = 105°C, Reaction volume = 100 µL, Run time = 25–40 min
    b. Step 1: 45 sec at 98°C
    c. Step 2: 20 sec at 98°C
    d. Step 3: 30 sec at 54°C
    e. Step 4: 20 min at 72°C
    f. Repeat steps 2–4 for 5–16 cycles (Cycle number is determined by cDNA input. Less input cDNA requires more cycles)
    g. Step 5: 1 min at 72°C
    h. Step 6: Hold at 4°C

Note: Samples can be stored at 4°C for 72 h, or used immediately.

97. Vortex the SPRIselect reagent to resuspend and add 60 µL to each sample and pipette fifteen times to mix.
98. Incubate for 5 min at 20–25°C and then place on the magnet until the solution clears.

99. Transfer 150 μL of each supernatant to a new PCR tube.

⚠ CRITICAL: DO NOT discard the supernatant.

100. Vortex the SPRIselect reagent to resuspend and add 20 μL to each supernatant and pipette fifteen times to mix.
101. Incubate for 5 min at 20–25°C and then place on the magnet until the solution clears.\
102. Remove and discard 165 μL of supernatant.
103. Add 200 μL 80% ethanol to the pellet and incubate for 30 sec.
104. Remove the ethanol and repeat the wash once more with 80% ethanol.
105. Centrifuge the tubes briefly, place the tubes back on the magnet, and remove the residual ethanol. Air dry for 2 min.
106. Remove samples from the magnet and add 35.5 μL Buffer EB to each sample, mixing by pipette fifteen times.
107. Incubate for 2 min at 20–25°C, then return the samples to the magnet until the solution clears.
108. Transfer 35 μL of each sample to new PCR tubes.
109. Store samples at 4°C for 72 hr, −20°C for long-term storage, or use immediately.
110. QC the libraries using the Agilent TapeStation.
    a. Analyze 2 μL of each sample using the Agilent D1000 High Sensitivity ScreenTape to obtain the concentration and average fragment size of each library.
111. See Figure 3 in expected outcomes Normalize each library to 3 nM and quantify by qPCR using the KAPA Library Quantification Kit – Illumina/ROX Low on the QuantStudio 12 K Flex Real-Time PCR System.
112. Pool libraries to create 3nM pools based on the concentration obtained from the qPCR.
    a. The number of libraries per pool is determined by the sequencing kit being used, the estimated number of expected cells per sample after GEM generation, and a target of 25,000 reads/cell.
113. Quantify the library pools by qPCR using the KAPA Library Quantification Kit – Illumina/ROX Low on the QuantStudio 12 K Flex Real-Time PCR System.
114. Sequence the libraries according to the NovaSeq 6000 System Guide. We did not test additional 10x sequencing kits, additional studies are needed to interrogate the impact of newer 10x genomics kits.

EXPECTED OUTCOMES

This protocol enables the liberation of mononuclear cellular suspensions with improved cellular recovery and viability for scRNA-seq. As an example, post Annexin V+ cellular depletion a significant decrease in mitochondrial cDNA (Figure 1A), improved cell recovery (Figure 1B), and genes detected (Figure 1C) were noted in the quality metrics from cryopreserved biopsies.

TapeStation Analysis software was used to calculate the defining regions for each sample (Figure 2). The average concentration and library size of the final library were calculated by defined regions for each library in the region settings of the TapeStation Analysis software as demonstrated in Figure 3.

LIMITATIONS

Despite our ability to recover many crypt-like stem cells with this protocol, limited expression of markers for the crypt small intestine Paneth cells, such as DEFA4 and DEFA6, was observed, suggesting that an epithelial-specific processing step may be required to adequately sample this limited cell type. Moreover, despite liberating a significant number of myeloid cells with this protocol, substantial patient-to-patient variation was observed within the myeloid compartment.

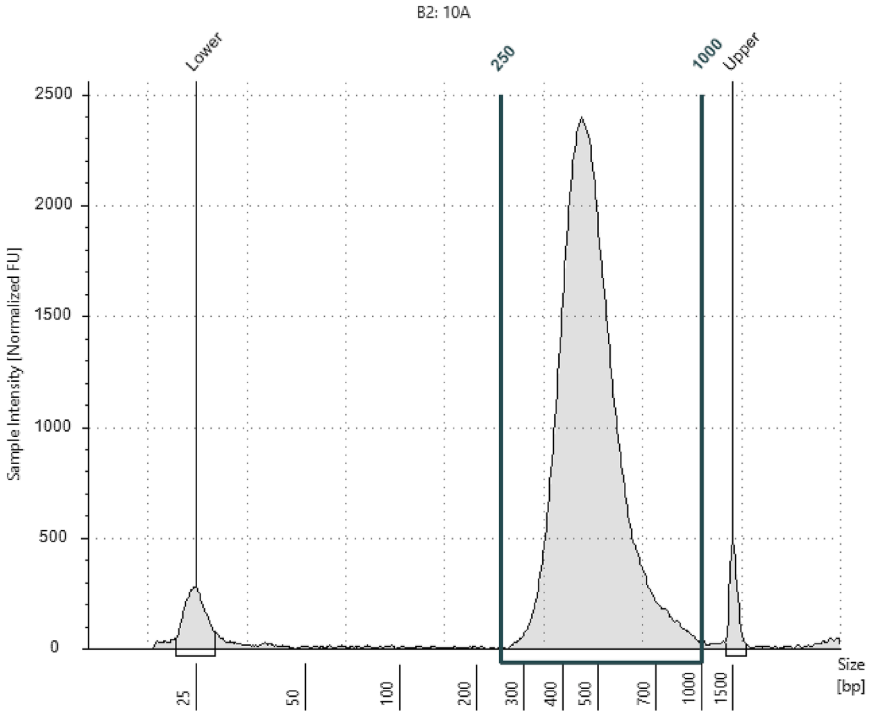


Figure 3. Representative trace from the final library QC

The concentration (Conc. [pg/mL]) and average library size (Average Size [bp]) of each final library are calculated by defining regions for each library in the Region Settings of the TapeStation Analysis software. Regions are defined using a From [bp] and a To [bp] as shown in the graph. In the graph, the From [bp] = 250 and the To [bp] = 1000. The concentration and average library size can be obtained from the Region Table tab in the software.

This protocol did not test other versions of the NextGEM 10x Genomics kits beyond those outlined above, this is a critical consideration, as other versions may provide improved sequencing depth or different QC metrics.

The utilization of Annexin V–based depletion may remove some non-apoptotic immune cells and may not capture all mechanically damaged cells; this should be considered when using this protocol. Additionally, alternative approaches such as live-cell FACS (e.g., AO/PI) could further refine cell selection using this protocol.

This protocol utilized cryopreserved samples, we were unable to test his protocol using fresh endoscopic biopsies due to sample access.

TROUBLESHOOTING

Problem 1

Lower than expected cell yield. Expected yields after dead cell removal are 1,986–38,013 per single endoscopic biopsy.

Potential solution

- Before thawing, inspect the biopsy vial to determine that the biopsy is fully immersed in cryostor. If not immersed, improper cryopreservation could explain the result.
  - Confirm that sample has been placed in cryostor immediately after endoscopy and cryopreserved correctly.

- After thawing, check the sample's size and quality. Some samples may have proportionally greater fat and connective tissue.
- Make sure that digestion buffers have not expired and have not undergone multiple freeze-thaw cycles.
- Increase mincing time with scissors if there are pieces > 1mm.
- For aspiration steps after washing, be careful not to touch the cell pellet.

Problem 2

Low viability after dead cell removal.

Potential solution

- Before thawing, inspect the biopsy vial to determine that the biopsy is fully immersed in cryostor. If not immersed, improper cryopreservation could explain the result.
  - Confirm that sample has been placed in cryostor immediately after endoscopy and cryopreserved correctly.
- Ensure that the processing time is less than 3 hrs. Reduce the number of samples processed if needed.
- Check that centrifugation speeds do not exceed 300 g.

Problem 3

Cell clumping.

Potential solution

- Ensure that DNase has not expired and that aliquots have not undergone more than one freeze-thaw cycle.
- After DNase is washed out (step 20), minimize the time spent on further processing.
- After the dead cell removal step, keep the cells at 4°C.

Problem 4

Uneven emulsion volume during GEMs transfer from the chip indicates a reagent clog in the well containing the lower volume.

Potential solution

- Ensure Gel Beads are stored at −80°C.
- Ensure Gel Beads are completely thawed, vortexed, and centrifuged before loading onto the chip.
- Ensure cells are not clumped and are free from debris.
- If cells and reagents are remaining, repeat GEM generation.

Problem 5

The opacity of an emulsion is not the same as the others during GEMs transfer from the chip, indicating a wetting failure.

Potential solution

- Ensure reagents were loaded into the correct well on the chip.
- Ensure reagents were added in the correct order.
- Avoid introducing air bubbles while loading the chip.
- If cells and reagents are remaining, repeat GEM generation.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Darren Ruane (druane@its.jnj.com).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Daniel Horowitz (dhorowit@its.jnj.com).

Materials availability

All materials requests will be fulfilled by technical contact.

Data and code availability

The dataset generated during the current study is publicly available on https://www.ncbi.nlm.nih.gov/ (accession number: GSE277276). All analytical methods, including statistical codes, software information, and algorithms used in this study, will be made available upon request to the lead contact.

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AUTHOR CONTRIBUTIONS

D.H., J.R., L.T., and T.L. performed the experiments and analyzed the data. D.H., J.R., L.T., T.L., A.K., and D.R. contributed to the writing of the manuscript. A.K. and D.R. provided research funding, research supervision, or research materials. D.H., J.R., A.K., and D.R. conceived the research, led the project, analyzed the data, interpreted the results, and wrote the manuscript.

DECLARATION OF INTERESTS

J&J authors are employees of Janssen Research & Development, LLC, a wholly owned subsidiary of Johnson & Johnson, and may own stock in Johnson & Johnson.

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