Oct 26, 2023

Sputum Sample Processing for Single Cell Isolation and Live Recovery for Single Cell RNA Sequencing

DOI
https://dx.doi.org/10.17504/protocols.io.j8nlko9k5v5r/v1

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Protocol Citation: Jason D Limberis, Alina Nalyvayko, Michele Tomasicchio, Shameem Jaumdally, anil pooran, Keertan Dheda, john.metcalfe 2023. Sputum Sample Processing for Single Cell Isolation and Live Recovery for Single Cell RNA Sequencing. **protocols.io** https://dx.doi.org/10.17504/protocols.io.j8nlko9k5v5r/v1

Protocol status: Working
We use this protocol and it's working

Created: September 11, 2023

Last Modified: October 26, 2023

Protocol Integer ID: 87649

Keywords: sputum, single cell isolation, single cell RNA sequecncing, scRNAseq, infectious disease, clinical sputum samples for single live cell isolation, live recovery for single cell rna sequencing processing, single cell rna sequencing processing, single live cell isolation, clinical sputum sample, single cell, rna, cell, live recovery

Funders Acknowledgements:
UCB Catalyst
Grant ID: Chipotle

Abstract

Processing of clinical sputum samples for single live cell isolation for downstream applications.

Materials

- Zymo DNA/RNA Shield **Fisher Scientific Catalog #**50-125-1706
- Phosphate buffer solution (PBS)
- EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #**AM9260G
- Bovine Serum Albumin (BSA) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**A7906
- Magnesium Chloride **Fisher Scientific Catalog #**AC223210010
- DMSO **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**472301
- Fetal calf serum (FCS) **Gemini Bio-Products Catalog #** 900-108
- DNase I, Bovine Pancreas, >500 Kunitz U/mg **Gold Biotechnology Catalog #**D-300
- 100 g N-Acetyl-L-Cysteine **biorbyt Catalog #**orb320385
- Collagenase, Type IV, powder **Thermo Fisher Catalog #**17104019

Before start

Increasing the amount of sputum, by asking the patient to expectorate several time, will likely increase the final cell numbers.

Prepare Buffers

1

5mM EDTA in PBS

500ul 0.5M EDTA stock

49.5ml PBS. Store at 4C

10% BSA stock

Add 1g BSA to 10ml PBS, mix gently till dissolved

Filter sterilize & keep at 4C

50mM MgCl2 solution

Add 238mg of MgCl2 to 50ml of H2O

10% DMSO/FCS freezing mixture

5 mL DMSO

45 mL fetal calf serum (FCS)

[M] 7.5 Mass Percent **NALC in PBS**

DNase

Aliquoted into 100ul aliquots at 224kuml and stored at -20C

Add 100ul to 40ml dissociation buffer to make a final conc of 0.56ku/ml.

Collagenase IV

Add 500 µL to 40 mL dissociation buffer to make a final conc of 0.05mg/ml

Aliquoted 500 µL at 4mg/ml and stored at -20C.

Make fresh for each use

1% BSA in PBS

5 mL 10% BSA

45 mL PBS

Dissociation buffer

1.6 mL of [M] 50 millimolar (mM) $MgCl_2$

100 µL DNase

500 µL collagenase

37.8 mL PBS

Sputum collection

2 Collect an induced sputum, preferably 5 mL or more in volume, and process as below as soon as possible.

2.1 Weigh the sputum collection cup before and after the sputum collection.

2.2 Estimate the volume of sputum collected using a graduated container of the same size.

2.3 Record the viscosity (salivary, mucosalivary, purulent, mucopurulent)

2.4 Record the time of sputum collection

Bulk RNA sequencing

3 Record the time of sputum processing

4 Using a wide bore tip (or cut the bottom of a 1000 µL) draw up ~ 200 µL sputum. If the sample is too viscous for a pipette, use a syringe needle instead.

5 Add to a 1.5ml tube containing 500 µL Zymo RNA Shield

6 Vortex tube for 00:00:10 10s

7 Immediately transfer to -80 °C

Make a single cell suspension (scRNAseq)

8 Add 40 mL dissociation buffer into the collection tube containing the sputum sample

9 Secure cap, manually invert the tube several times. Secure to a hula mixer (low setting) and incubate for 00:10:00 at Room temperature . 20m

Note: Big clumps should dissolve after 00:10:00 . Some small fibers may will remain.

10 Add DTT to a final concentration of 1-2mM. 5m
Invert the tube several times and incubate for 00:05:00

11 Pass the dissociated sample through a 100 µm strainer on top of a new 50 mL tube using an automated pipettor (do not pour). 350 rcf, 4°C, 00:10:00 10m

12 Wash the strainer with 2 mL of PBS

13 Spin tube in a sealed bucket at 250 rcf, 4°C, 00:10:00 10m

14 Remove 1000 µL supernatant and place in a cryovial for Luminex Cytokine assays.
Add 140 µL of protease inhibitor and store at -80 °C

15 Aspirate the remainder of the supernatant and discard.

16 Gently resuspend cell pellet in 1 mL EDTA/PBS using a wide bore 1000 µL tip

17 Count cells using Turks protocol followed by paraformaldehyde fixation to decontaminate

Long-term storage of cells (scRNAseq)

18 Pre-cool CellCool and cryovials at 4 °C for > 02:00:00 2h

19 Place cells On ice

20 Gently mix the cells

21 Centrifuge at 300 rcf, 4°C, 00:05:00 5m

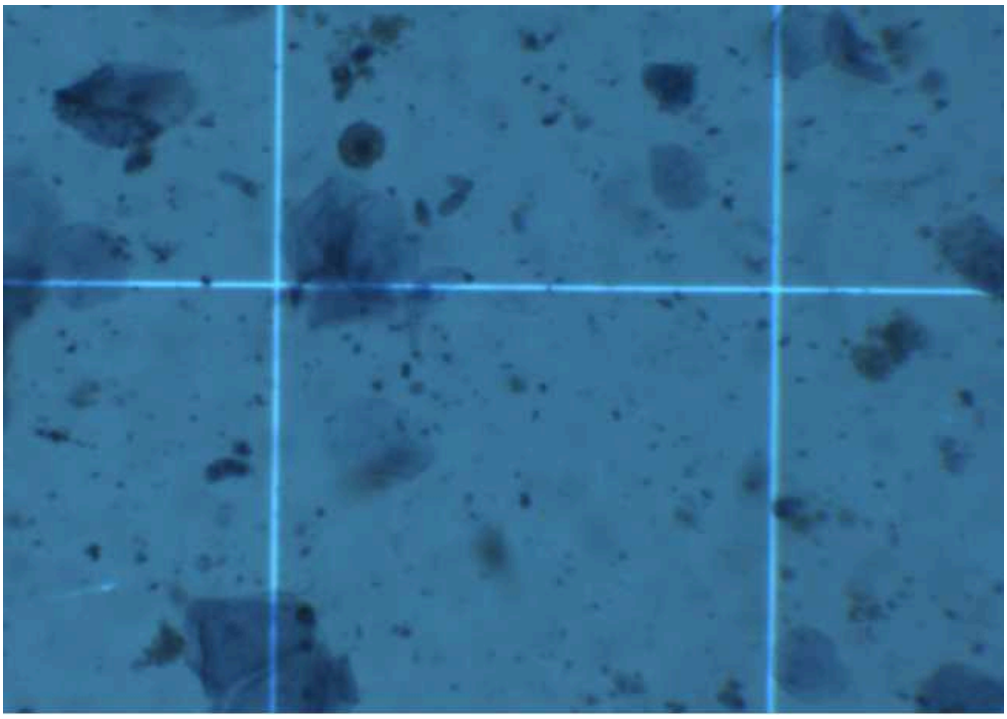
22 Discarded supernatant and resuspend cell pellets in pre-chilled (4 °C) 10% DMSO/FCS solution (add dropwise)

23 Dispense cell suspension in 500 µL aliquots into the pre-cooled cryovials and place into CoolCell at -80 °C

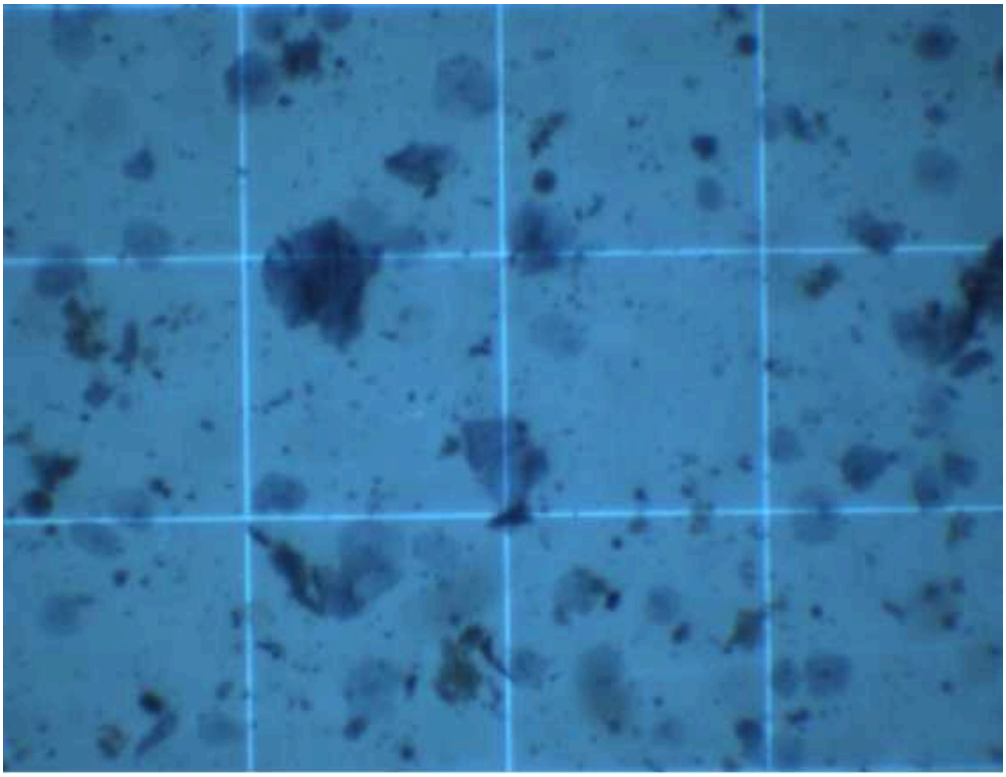
Expected results

24 **Pilot samples**

	A	B	C	D
	Sample	S1	S2	S3
	Viscosity	mucos alivary	mucos alivary	mucos alivary
	Volume used (ml)	10	4	6
	Total cells before freezing (10^6)	2.2	0.9	2.9
	Total cells recovered after thawing (2 weeks after frozen; 10^6)	0.88	0.48	1.33
	Percentage cell recovery post freezing (%)	40	53	46



S2 400x



S3 100x

Clinical samples

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
	Sample ID	Time to sputum processing (h.m)	weight of sputum (g)	volume of sputum (ml)	viscosity of sputum	Time to thaw (days)	Total cell count pre freeze (x10^6)	Total cell count taken forward pre freeze (x10^6)	Total cell count post freeze (x10^6)	Total cell count difference (x10^6)	Live cell count	Dead cell count	Viability (%)	Viability of pre freeze total (%)
	CC20	2.85	17.8	15	salivary	662	9.1	7	4.86	2.14	1.58	3.28	33	23
	CC22	<6.00	2.1	2	mucoid	658	1.3	1.3	0.60	0.71	0.15	0.44	26	12
	CC23	2.10	2.9	3	mucosaliv ary	657	0.43	0.43	0.37	0.06	0.08	0.29	21	18
	CC24	1.10	8.7	8	mucosaliv ary	657	1.1	1.1	0.81	0.30	0.19	0.62	23	17
	CC25	4.90	0.7	0.9	mucosaliv ary	652	0.17	0.17	0.00	0.17	0.00	0.00	0	0
	CC26	2.00	1.5	1.4	mucosaliv ary	652	1.13	1.13	0.73	0.40	0.17	0.57	23	15
	CC27	2.20	3.2	3	mucosaliv ary	651	1.4	1.4	1.33	0.07	0.30	1.03	23	22
	CC28	2.00	12	10	mucoid	644	3.2	2	2.74	-0.74	0.29	2.45	11	15

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
	CC33	2.00	3	3	mucosaliv ary	627	0.8	0.8	0.67	0.13	0.25	0.42	37	31
	CC37	<6.00	4.1	4	mucosaliv ary	623	0.92	0.92	0.97	-0.05	0.34	0.63	35	37