Multi tissue processing for single cell sequencing of human immune cells

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Abstract

This protocol has been developed for the simultaneous processing of multiple human tissues to extract immune cells for single cell RNA sequencing using the 10X platform, and ideal for atlasing projects. Included in this protocol are the steps needed to go from tissue to loading the 10X Chromium for single cell RNA sequencing and includes the hashtag and CiteSeq labelling of cells as well as the details needed to stimulate cells with PMA+I.

Keywords

Human, tissue, single cell RNA sequencing, Atlas

Materials

Reagent	Supplier	Part No.
X-vivo 15	Lonza	BE02-060Q
FBS	Merck	F7524-500ML
PBS (No Ca^{2+} or Mg^{2+})	Merck	D8537-500ML
7.5% BSA	Gibco	15260-37
0.5M EDTA	Invitrogen	15575-038
DTT powder 0.1 M DTT solution	Roche Thermo	10197777001 707265ML
Collagenase IV	Merck	C7926-100MG
Dispase	Sigma	D4693-1G
Benzonase	Merck	70746-4
Ficoll	GE Healthcare	17-1440-03
Cell stim cocktail (PMA+I)	eBioscience	00-4970-93
Hashtags	Biolegend	Total-C
CITE-Seq 130 panel	Biolegend	399905

Flowmi 40μM filters	Sigma	H13680
2.5ml syringe	Appleton Woods	GS574
Cell strainers 70μM	Corning	352350
Gentlemacs C tube	Miltenyi	130-093-237

Solutions to make:

Solution	Base	Reagent 1	Reagent 2
X-vivo + 1% FBS	49.5 ml x-vivo	0.5 ml FBS	2.5 μl Benzonase
PBS + 0.04% BSA	500 ml PBS	2.66 ml 7.5% BSA	
X-vivo +5 mM EDTA + 2 mM DTT + 1% FBS	48.5 ml x-vivo + 1% FBS	0.5 ml of 0.5M EDTA	1 ml of 100 mM DTT

Equipment

In addition to the regular equipment found in a Containment level 2 laboratory you will need:

Miltenyi Gentlemacs
37ºC incubator
10X Chromium instrument

Citations

The gut processing protocol has been taken from:

James, K.R., Gomes, T., Elmentaite, R. et al. Distinct microbial and immune niches of the human colon. Nat Immunol 21, 343–353 (2020). https://doi.org/10.1038/s41590-020-0602-

The skin processing protocol has been taken from:

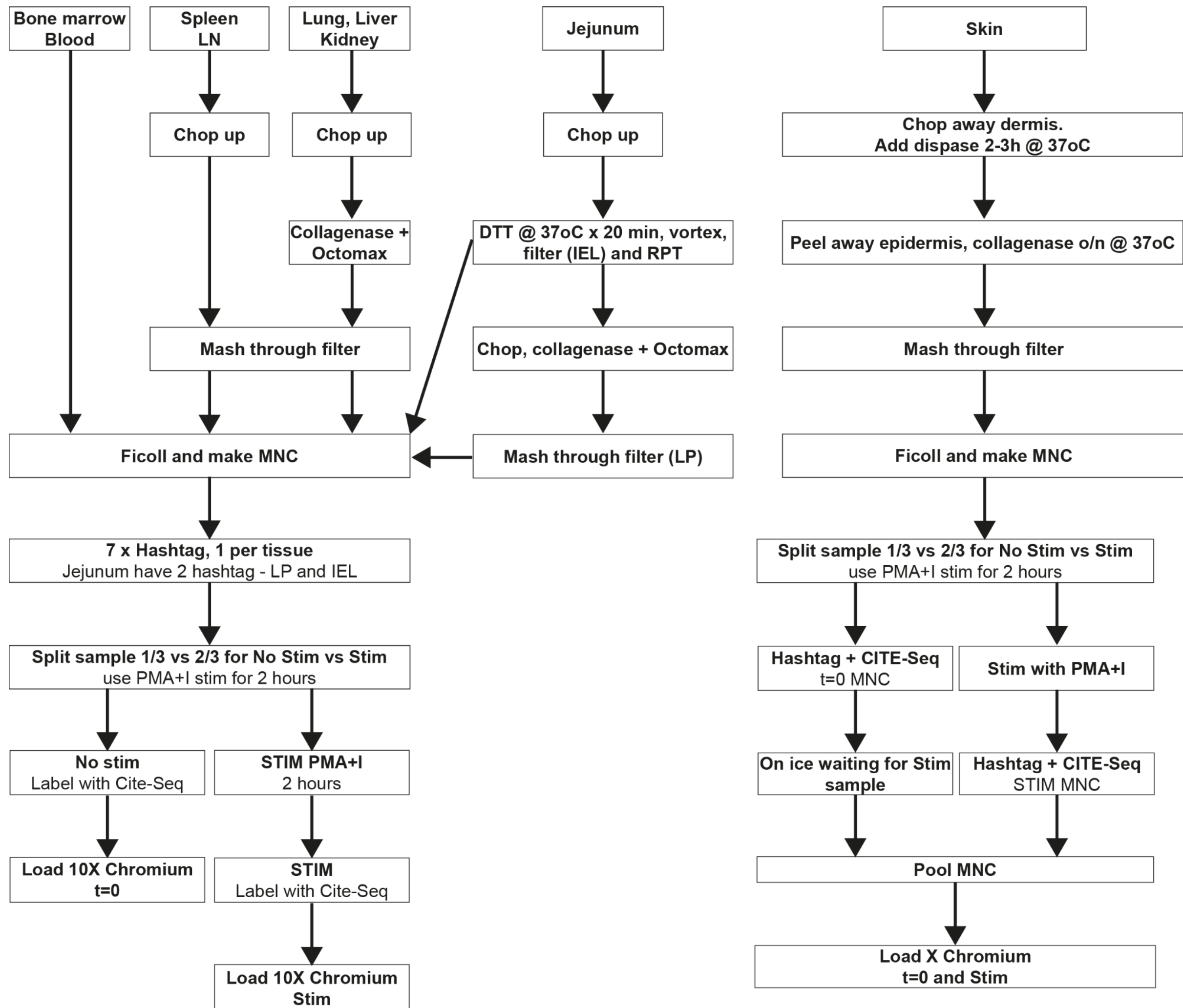
Human skin single cell dissociation on Protocols.io
https://www.protocols.io/view/human-skin-single-cell-dissociation-ripd4dn

Additional notes

This protocol has been optimised for extracting immune cells from small pieces of tissue (around 10g or less) and has divided tissues into five categories depending on how much mechanical or chemical digestion is needed to enable extraction of immune cells, as shown in the workflow diagram. Blood and bone marrow need no processing. Lymphoid tissues like spleen and lymph nodes require a gentle mashing to make a cell suspension. Non-lymphoid tissues like lung, liver and kidney require both a mechanical and collagenase digestion. The gut and skin require more specialised protocols and use published protocols from the James and Hanifa laboratories.

We include an activation step using PMA+I for 2 hours, however this stimulation condition will need optimising depending on the hypothesis being tested.

Workflow



Tissue to cell suspension

Bone Marrow and Blood

1. No processing, go straight to ficoll layering.
2. Place on ice until other tissues have caught up.

Lymphoid Tissues (Spleen, Lymph node)

1. Mash the lymphoid tissue through a 70 µM filter placed on top of a 50ml falcon, using the plunger from a 2 ml syringe as a pestle.
2. Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.
3. Depending on the size of the tissue, top up the filtered cell suspension to 30-50ml with x-vivo + 1% FBS.
4. Place on ice until other tissues have caught up.

Non-lymphoid tissue (Lung, Liver, Kidney)

1. We receive around 5g of tissue and the protocol will need to be scaled up if more tissue is being processed. Do not overload the Gentlemacs as this will reduce cell yield.
2. Chop up the tissue with scissors into 0.2-0.5cm pieces.
3. Do not overload the Gentlemacs, with no more that 5
4. Transfer to Gentlemacs tube and add 2.5ml of collagenase and 2.5 ml x-vivo.
5. Run the following programme that takes 32 minutes
    a. Loop 3 times:
    b. Ramp 900 rpm 12 sec
    c. Spin 700 rpm 1 sec
    d. Ramp 1000 rpm 8 sec
    e. Spin 1500 rpm 1 sec
    f. Spin 1900 rpm 4 sec
    g. Spin 1500 rpm 1 sec
    h. Spin 1900 rpm3 sec
    i. Temp on @ 37ºC and loop 2 times:
    j. Spin 50 rpm 15 min
    k. Spin 350 rpm 20 sec
6. Add 20 µl of 0.5 mM EDTA (2mM final conc) per 5 ml of collagenase to neutralise and shake to mix.
7. Pour and scrape digested tissue into a 70µM cell strainer placed on top of a 50 ml falcon.

8. Use the plunger of a 2 ml syringe to mash tissue through the filter, like a pestle.
9. Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.
10. Depending on the size of the tissue, top up the filtered cell suspension to 30-50ml with x-vivo + 1% FBS.
11. Place on ice until other tissues have caught up.

Jejunum

- **Protocol adapted from from Kylie James "Distinct microbial and immune niches of the human colon", Nature Immunology, 2020.**

1. We receive around 5g of tissue and the protocol will need to be scaled up if more tissue is being processed. Do not overload the digestion steps as this will reduce cell yield.
2. Wash jejunum with PBS + 0.04% BSA to remove any chime.
3. Chop up the jejunum with scissors into 0.5 cm pieces.
4. Transfer to a 50 ml falcon tube and add 10 ml of x-vivo + 2 mM DTT + 5 mM EDTA + 1% FBS and put in the 37ºC incubator for 20 minutes and shake after 10 minutes.
5. Put jejunum chemical digest through a 70μM filter on top of a 50 ml falcon and rinse with 10 ml of x-vivo + 1% FCS.
6. The wash through from the filter contains the **IEL cells**, keeping the falcon on ice.
7. Scrape tissue from the filter back into a 50 ml falcon and repeat the digest with 10 ml x-vivo + 2 mM DTT + 5 mM EDTA + 1% FBS and place back in the 37ºC incubator for 20 minutes, and shake after 10 minutes.
8. Put jejunum digest through a 70μM filter on top of the 50ml falcon containing the **IEL cells** and rinse with 10 ml of x-vivo + 1% FCS. Keep the IEL cells on ice.
9. Scrape tissue from the filter into a Gentlemacs C tube and digest with 2.5 ml of collagenase and 2.5ml of x-vivo and run the programme called 'Sarah' takes 32 minutes, with various mixing speeds.
10. Add 20 μl of 0.5 mM EDTA (2mM final conc) per 5 ml of collagenase to neutralise and shake to mix.
11. Pour and scrape digested tissue into a 70μM cell strainer placed on top of a 50 ml falcon.
12. Use the plunger of a 2 ml syringe to mash tissue through the filter, like a pestle.
13. Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue, cells that pass through the filter are **LP cells**.
14. Depending on the size of the tissue, top up the filtered cell suspension to 30-50ml with x-vivo + 1% FBS.
15. Place on ice until other tissues have caught up.

Skin

- **Protocol from Haniffa Lab, Newcastle University**
  https://www.protocols.io/view/human-skin-single-cell-dissociation-ripd4dn

Depending on when tissues arrive, skin can either be set up the night before and then will be processed with all the other tissues. Or will have to be processed the next day.

- If processed the same day as other tissues then will be hashtagged with all other tissues.
- If processed the next day will have to follow the same procedure but the unstim will wait on ice until the stim catches up and can be loaded on 1 lane of 10x.
    - 5μl of CITE-Seq will need to be left from the processing of the other tissues.

1. Chop into ~0.5cm^2 sized pieces. Remove as much dermis from each as possible using a razor blade - be careful, extremely sharp. Discard the dermis layer.
2. Incubate the retained skin in dispase for 2-3 hours at 37ºC, to allow the epidermis to be stripped.
3. Separate the epidermis from the dermis using fine forceps. These can be kept separate or processed together.
4. Wash in PBS.
5. Add collagenase at 3X the volume of the tissue and incubate at 37˚C overnight.
6. Add 20 μl of 0.5 mM EDTA (2mM final conc) per 5 ml of collagenase to neutralise and shake to mix.
7. Scrape the digested skin and media into a 70μM filter on top of a 50 ml falcon.
8. Use the plunger from a 2 ml syringe to mash the skin through the filter, like a pestle.
9. Occasionally wash the filter with x-vivo + 1% FBS as you mash the tissue.
10. Depending on the size of the tissue, top up the filtered cell suspension to 30-50ml with x-vivo + 1% FBS.
11. Place on ice until other tissues have caught up, or if processing the next day alone proceed with cell count and ficoll.

Cell suspension to MNC

Wash cell suspension

1. Once all the tissues have reached a cell suspension, spin at 600g x 10 minutes.
2. Pour off supernatant and resuspend in x-vivo + 1% FBS, the volume to resuspend depends what you are going to layer over ficoll.
3. There is no exact science to the layering but as a guide:
    a. Spleen - 90 ml
    b. Lymph nodes - 7 ml
    c. Non-lymphoid tissue - up to 60 ml
    d. Skin - 7 ml

Ficoll

Depending on the size of the tissue and cell pellet depends on how many ficoll tubes you need.

- Bone marrow 10ml + 20 ml x-vivo layered on 15ml ficoll per 50ml falcon.
- Blood up to 15ml + 15 ml x-vivo layered on 15ml ficoll per 50ml falcon.
- Spleen 30 ml cells suspension over 15ml ficoll per 50ml falcon x3.
- Lymph nodes 7 ml cells suspension over 8 ml ficoll per 15 ml falcon.

- Non-lymphoid tissue depending on the size of the cell pellet up to 30 ml cell suspension over 5 ml ficoll x2.
- Skin 7 ml cells suspension over 8 ml ficoll per 15 ml falcon.
- Spin tubes at 400g for 25 minutes with slow deceleration. Takes around 40 minutes to run.

CD66b and RBC depletion

1. It is good to remove granulocytes (expressing CD66b) and RBC from each sample where required as these cells do not provide useful single cell sequencing information.
2. Use Stem Cell CD66b positive selection kit (17882) to remove granulocytes from each sample and Stemcell RBC depletion reagent (18170).
    a. In a 15 ml falcon, add between 0.5 to 3ml of sample (up to 5 million cells).
    b. Add 25 µl of CD66b positive selection cocktail, mix and incubate for 3 minutes at room temperature.
    c. Vortex RapidSpheres for 30 seconds.
    d. Add 25 µl of RapidSpheres, mix and incubate for 3 minutes at room temperature.
    e. Add 25 µl of RBC depletion reagent per 1 ml of sample and mix.
    f. Immediately place the samples on a magnet for 5 minutes.
    g. Carefully pipette off the supernatant to a fresh tube and place on ice. This contains the cells you want, DO NOT discard.
    h. Wash the beads with 5 ml of PBS 1% FBS + 1 mM EDTA and place back on the magnet for 5 minutes.
    i. Collect supernatant and add to the fresh tube in part (g).
    j. Throw away the leftover tube with beads as this contains the granulocytes and RBC.

Count cells

1. Count cells from each tissue after ficoll (and CD66b / RBC depletion).
2. Make sure cells are well mixed and count with trypan blue. If count all 25 squares of the haemocytometer, then:
3. Cell count x dilution factor x volume x 10,000 = Total cell count.

Hashtag, CITE-Seq and stimulation

Hashtag

4. Take at least 500k MNC per tissue (use 750k to 1 million cells if available) into a 1.5ml lo-bind eppendorf.
5. Spin cells at 600g for 5 minutes, remove as much supernatant as possible and resuspend in 50µl PBS+0.04% BSA.
6. Record which hashtag is used for which tissue.
7. Add 5µl FC block and incubate at 4ºC for 10 minutes.
8. Spin each hashtag at 14,000g for 10 minutes.

9. Add 0.5µl of hashtag to each tube.
10. Incubate at 4ºC for 30 minutes.
11. **(Make up lyophilised CITE-Seq antibodies - section CITE-Seq section)**
12. Top up to 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
13. Wash cells with 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
14. Resuspend in 100µl of PBS + 0.04% BSA.

Count cells

Count cells from each tissue after the Hashtag washes as there will be cell loss, and if a particular tissue has fewer cells than needed, then repeat the hashtag process with more cells.

Make sure cells are well mixed and count with trypan blue. If count all 25 squares of the haemocytometer, then:

Cell count x dilution factor x volume x 10,000 = Total cell count.

Pool MNC from all tissues and split for Unstim and Stim (if required)

1. Use the post hashtag cell counts to pool MNC from each tissue at equal cell number, based on what the lowest count is, into a 1.5ml lo-bind eppendorf.
2. Ideally you want 300k - 400k from each tissue. Record the total volume.
3. Flick to mix the cells really well.
4. Remove ⅓ of the cell volume to a new 1.5 ml tube and label as Unstim and top up to 500µl with PBS + 0.04% BSA. Spin at 600g x 5 min and proceed to the CITE-Seq section.
5. To the remaining ⅔ of pooled MNC, label the tube as Stim and top up to 1 ml with x-vivo + 1% FCS and proceed to MNC stimulation.

MNC Stimulation with PMA+I

1. We are using a PMA+I stimulation which we have optimised to assess early activation events, and depending on the hypothesis being tested may need to be a different stimulant and time point.
2. Get the MNC stim on as it takes two hours.
3. Culture pooled MNC in 1ml of x-vivo + 1% FBS for 2 hours at 37ºC with 2µl of cell stim cocktail (PMA+I). Flick tube to mix cells every 30-40 minutes.
4. Culture MNC at no more than 2 million cells per ml. Use more than one 1.5ml tube if needed. Cell stim cocktail (PMA+I, eBioscience) is 1:500 stock.
5. After two hour incubation, move to **Cite-Seq of stimulated cells**.

CITE-Seq

1. Make up lyophilized CITE-Seq antibodies - each vial is enough for 500k cells, but will use 1 vial for up to 2 million cells.
    a. Spin lyophilised reagent at 10,000g for 30 seconds.

b. Add 27.5µl Cell staining buffer to the lyophilised CITE-Seq reagent and briefly vortex.
c. Incubate at RT for 5 minutes.
d. Briefly vortex again, then spin at 10,000g for 30 seconds.
e. Transfer entire volume to a lo-bind PROTEIN tube.
f. Spin at 14,000g for 10 min at 4ºC
g. Store in the fridge until ready to use.

2. Spin the unstim pool MNC at 600g x 5 minutes and remove supernatant.
3. Resuspend cells in 50µl of PBS + 0.04% BSA.
4. No need to add FC block, as already done at hashtag stage
    a. If not hashtagged already, then add 5ul FC block for 10 min at 4ºC.
5. Add 10µl of CITE-Seq 130Ab and incubate at 4ºC for 30 minutes.
    a. Keep the remaining CITE-Seq reagent for the stimulated sample and the skin if processed the next day.
6. **(Take 10x reagent out of the freezer to warm up to RT, during CITE-Seq incubation. It takes 30 minutes to warm up to RT.)**
7. Top up to 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
8. Wash cells with 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
9. Resuspend cells in 250µl PBS + 0.04% BSA and put through a flowmi filter. Rinse out 1.5ml tube with 250µl PBS + 0.04% BSA, and put this through the same Flowmi filter. **TIP** - use a second 1ml pipette tip, so can keep Flowmi filter on original tip, remove from pipette and pipette second 250µl wash into the top of the tip with the filter. Reattach the pipette and wash through the filter.
10. Spin at 600g x 5 mins, and remove supernatant.
11. Resuspend in 100µl of PBS + 0.04% BSA.

Count cells

1. Count the unstim pooedl MNC sample.
2. Make sure cells are well mixed and count with trypan blue.
3. 2µl of cells to 8µl of Trypan blue. If count all 25 squares of the haemocytometer, then:
4. Cell count x 5 x 0.1 x 10,000 = Total cell count.

Load unstim for 10x

1. Ideally you want to load cells at 1,000 cells per 1µl (Max 2,000 cells / µl).
2. Dilute the sample (if needed).
3. Load 15,000 cells per tissue, 30,000 cells per 10x GEM reaction.
4. So for 6 tissues, it would be 90,000 cells over 3 10x GEMs.

Cite-Seq of stimulated cells

1. After the 2 hour stimulation, spin the stim pool MNC at 600g x 5 minutes and remove supernatant.
2. Resuspend cells in 15 µl of PBS + 0.04% BSA.
3. No need to add FC block as already done at hashtag stage.

4. Add 12.5 µl of CITE-Seq 130Ab and incubate at 4ºC for 30 minutes.
5. **(Take 10x reagent out of the freezer to warm up to RT, during CITE-Seq incubation. It takes 30 minutes to warm up to RT.)**
6. Top up to 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
7. Wash cells with 500µl with PBS + 0.04% BSA, and spin at 600g x 5 mins, and remove supernatant.
8. Resuspend cells in 250µl PBS + 0.04% BSA and put through a flowmi filter. Rinse out 1.5ml tube with 250µl PBS + 0.04% BSA, and put this through the same Flowmi filter. **TIP -** use a second 1ml pipette tip, so you can keep Flowmi filter on the original tip, remove from pipette and pipette second 250µl wash into the top of the tip with the filter. Reattach the pipette and wash through the filter.
9. Spin at 600g x 5 mins, and remove supernatant.
10. Resuspend in 100µl of PBS + 0.04% BSA.

Count cells

1. Count the Stim pooled MNC sample.
2. Make sure cells are well mixed and count with trypan blue.
3. 2µl of cells to 8µl of Trypan blue. If count all 25 squares of the haemocytometer, then:
4. Cell count x 5 x 0.1 x 10,000 = Total cell count.

Load stim for 10x

1. Ideally you want to load cells at 1,000 cells per 1µl (Max 2,000 cells / µl).
2. Dilute the sample (if needed) in PBS + 0.04% BSA.
3. Load 15,000 cells per tissue, 30,000 cells per 10x GEM reaction.
4. So for 6 tissues, it would be 90,000 cells over 3 10x GEMs.

Flow, Freezing and RNA from remaining cells

Remaining cells put in RLT

1. When all the 10x GEMs have been processed and they look good, pellet any leftover pooled unstim or stim MNC at 600g x 5 minutes and take off supernatant.
2. Flick to resuspend dry pellet and resuspend in 350µl of Qiagen RLT buffer.
3. Quickly vortex and freeze at -80ºC until ready to extract the RNA.

Flow cytometry

1. Run a flow panel to QC the sample and get proportions of the major cell types. Stain ~500k per tissue with the desired panel of antibodies. This is an example flow cytometry panel, however may need to be adjusted depending on the flow cytometer available:
    a. CD3 - Percp Cy5.5
    b. CD19 - APC
    c. CD56 - PE

   d. CD4 - PE Cy7
   e. CD14 - FITC
   f. CD16 - BV421
   g. CD8-APCCy7
2. Fix cells and store at 4ºC until they can be analysed.

Freeze down excess cells

1. Any cells that are not going for 10x or flow cytometry can be frozen down.
2. Spin cells at 600g x 5 mins, and remove as much supernatant as possible.
3. Flick to resuspend cell pellet.
4. Add cell freezing media dropwise, until ~ 10 million cells per ml.
5. Flick to mix, and transfer to labelled NUNC tubes.
6. Put NUNC tubes in a Mr Frosty and store at -80ºC overnight.
7. Next day, transfer to LN2 storage.