Gordon's single cell transcriptomics protocol

August 2018 YE, ultimately adapted from Picelli *et al.* 2014 *Nature Protocols*

1. Prepare cell lysis buffer

19 µL 0.2% (v/v) Triton X-100 solution
1 µL RNase inhibitor (eg RNase OUT)

aliquot 2µL per 200µL PCR tube
(do extremely cleanly!)

- can store at 4C for ~6 months
- can alter to 18µL Triton X and 2µL RNase inhibitor; if using 4µL per tube for very large organisms, double reactions downstream

2. Picking cells:

prepare:

lysis buffer from step 1
liqN2 or at least -80C
clean thin glass pipettes and cell picking apparatus (rubber tubing with tips)
inverted microscope with imaging capability
50-60mm coverslips ideal but not required
metal plunge-y holder for cryo tubes
pre-labelled cryo tubes

Isolate cells, wash them 2-3x in appropriate sterile medium, insert picked cell with minimal volume of fluid into PCR tube with lysis buffer and IMMEDIATELY place in cryo tube and dunk in liqN2 (or -80C; eg into ziplock and then into isopropanol pre-cooled to -80). Ensure cell is happy and moving right before freezing. Image either in sample or right before freezing depending on organism's wants.
If pooling cells, either pick multiples at once (quickly!), or take out tube from liqN2 and add in series. Keep track of images corresponding to each cell and tube!

For tricky organisms (eg anaerobes): filter sterilise native medium through 0.22µm syringe filter and use to wash cells.

remember to take out and store in -80C if not proceeding to step 3 right away

3. Lysis and reverse transcription

take out/prepare:

ice
clean PCR tubes
sterile sleeve things *pack extra*
oligo-dTs
reverse transcriptase (Superscript II)
RNase OUT
RT mix (prepared in advance) <-- make sure already prepared before starting
Enzyme mix (prepared fresh!)
dNTP mix

Clean workspace with RNase AWAY / equivalent anti-RNase cleaner (BRING WITH!)

start PCR programme 1:

PCR programme 1	
3 min	72˚C
pause	4˚C

Make sure all reagents thawed before getting cells!

thaw cell completely; add freeze-thaw cycle(s) if applicable (liqN2 / RT or handwarmth)
this part must be done quickly and extremely cleanly!
note: tiny volumes thaw in ~30s, do not let sit

add to each tube:
1µL oligo dT
1µL dNTP mix

quickly vortex, spin down, and *immediately* proceed to PCR programme 1. (keep on ice if waiting for lid to heat up)

while PCR programme 1 is running, prepare the enzyme mix:

"Enzyme mix" 2.2x (2rxns)	
1.1 µL	Superscript II
0.55 µL	RNase OUT
	use 0.75µL per rxn

"RT mix"	10.5x
FS5X	**21 µL**
Betaine (5M)	**21 µL**
DTT	**5.25 µL**
MgCl2 (0.5M)	**1.26 µL**
dH2O (ultrapure)	**2.42µL**
TSO	**1.05 µL**
	(51.98 µL total volume)

can survive ~5 freeze-thaw cycles (store at -20C); use 4.95µL per rxn

remove PCR tubes from cycler and place back on ice.

to each tube, add **4.95µL** RT mix and **0.75µL** enzyme mix. Start PCR programme 2.

pipette up and down or vortex, spin, immediately proceed to PCR programme 2 or store on ice while waiting for lid to heat.
(allow ~2.5h for this programme – possible stopping point)
*now the material is in cDNA form and not as sensitive to degradation (but still sensitive to contamination!)

	PCR programme 2	
RT and template-switching	90 min	42˚C
unfolding RNA secondary structures	2 min	50˚C
continuation of RT and template switching	2 min	42˚C
10 cycles	goto 2	10x
enzyme inactivation	15 min	70˚C
hold	pause	4 ˚C

4. PCR

Prepare:

- KAPA HiFi HotStart ReadyMix (2x)
- IS PCR primers (10µM)
- ultrapure dH2O
- clean PCR tubes

mix fresh each time: (right before taking out cDNA)

PCR mix	2.2x	4.4x
KAPA HiFi HotStart ReadyMix (2x)	**27.5 µL**	**55 µL**
IS PCR primers (10µM)	**0.55 µL**	**1.1 µL**
nuclease-free dH2O	**4.95 µL**	**9.9 µL**
total volume	(33 µL)	(66µL)
		use **15 µL** per rxn

Add **15µL** of PCR mix to each tube. Vortex, spin, proceed to PCR programme 3.

PCR programme 3 (PCR)	
3 min	98˚C
20 s	98˚C
15 s	67˚C
6 min	72˚C
goto 2	19x
5min	72˚C
pause	4˚C

Allow 2.5h for this PCR programme. Can alter number of cycles – trade-off is quantity of final cDNA vs. PCR errors, Gordon normally uses 18-20 cycles
(more cycles for predicted fewer material, but not more than 20)

---not necessary to do in the field past this point---

6. Clean-up

take out:

- magnetic beads (at least 20 min in advance) – vortex VERY VIGOROUSLY just before use
- 1.5mL clean tubes
- RNA grade ethanol
- ultrapure dH2O
- magnetic stand

take out cDNA. Right after VIGOURSLY vortexing room-temperature magnetic beads, add **1:1** volume thereof (**25µL** in the standard case). Mix thoroughly by pipetting up and down (do not vortex). Let incubate for **8min** at RT.
Prepare 80% EtOH (fresh before use!): need 400µL per sample, allow **500µL**/sample.
place cDNA with beads on magnetic stand for **5min**
Keeping tube in the magnetic stand, CAREFULLY remove supernatant without disturbing the bead pellet.
Add **200µL** EtOH per sample – do not disrupt pellet (remain on magnetic stand)
incubate for **30s**
CAREFULLY remove EtOH and replace with second **200µL** EtOH wash
incubate for **30s**
Remove EtOH very carefully, then remove remainder liquid with p10
air dry by leaving tubes open (still on magnetic stand) in clean bench or underneath *extremely clean* lid
once cracks appear in bead pellet, reconstitute in **17.5µL** of ultrapure dH2O. Pipette up and down to completely dissolve pellet, and incubate off magnet for **2min**.
(label final storage tubes for cDNA)
Place back on magnetic stand for **2min**.
Leaving tubes on magnetic stand, CAREFULLY take out **15µL** and place in clean labelled storage tubes for cDNA. Do not use the remaining 2.5µL.

7. Quantification (QUBIT; HS dsDNA standard)

(see QUBIT protocol)
Bring cDNA to QUBIT. Add **1-2µL** to 199-198µL clean QUBIT buffer. Use as directed – measure each tube 3-5 times while rotating the tube. Take average.
More is better, but anything >0.2ng/µL (>100ng is very suspicious for a single cell)
Keep QUBIT samples for PCR and cloning

8. Cloning

set heating block to 42˚C at least an hour before cloning! Monitor temperature and water level.

IS PCR before cloning (per rxn):

EcoTaq 2x	**5µL**
IS primers	**1.2µL**
cDNA(QUBIT)	**3.8µL**

(total volume: 10µL)

IS PCR programme:

[[CHECK MACHINE]]

run out **3µL** on 1% agarose gel
we want to see smears (preferably not bands, and higher size)
anything above 400bp average is adequate

Prepare:

set heat block to 42˚C an hour in advance. Fill with water.
ice!
timer
place tube of LB in 42˚C
StrataClone Cloning Buffer
PCR product (from IS PCR)
StrataClone Vector Mix amp/kan

1µL of IS PCR product as input for cloning, use *half reactions* from StrataClone kit.
from StrataClone protocol:
in clean PCR tube, mix the cloning reaction (in that order):

1.5 µL StrataClone Cloning Buffer
1 µL PCR product (from IS PCR)
0.5µL StrataClone Vector Mix amp/kan

incubate at RT for **5 min**, then place on ice.
meanwhile, take out StrataClone SoloPack competent cells (be VERY gentle, keep on ice during transit, thaw on ice)
add 1µL of the above cloning reactions to half reactions (**25µL**) of competent cells. (can keep second half in original tube)
gently mix by flicking tube and swinging down; DO NOT VORTEX OR MIX BY PIPETTING!
incubate mixture on ice for **20 min** (no more than 30 min).
(check heating block for 42˚C and water level)
move tubes to 42˚C heat block for *exactly* **45 seconds**
return tubes to ice for **2 min**

add **250 µL** of pre-warmed LB to each tube.
ensure lids are closed well and place tubes horizontally on a 37˚C shaker for at least an hour (1.5-2h if plating on kanamycin)

meanwhile, spread **70µL** ampicillin and **40µL** 2% X-gal on **2** LB plates per reaction.
dry right-side up in 37˚C room
store IS-PCR and ligation reactions at 4˚C; -20˚C for long term storage.

Spread **10µL** and **50µL** of each of the transformants onto amp/X-gal LB plates while adding **50µL** LB to aid plating
incubate at 37˚C overnight, upside down

plates can be parafilmed and stored at 4˚C.

9. sequencing clones
pick (**white**, not blue!) 16 or more colonies per sample into **8µL** water, lyse by incubating in 3 or 4 cycles of: 95°C --> 40°C, stop at 10°C (Clone-LY or Clone1)

Set up PCR and run programme Clone2:

Screening PCR
(per reaction)

EcoTaq 2x	**10µL**
T3	**1µL**
T7	**1µL**
colony	**8µL** colony in water

avoid bands <400bp as those are mostly insert

to sequence: submit unpurified, specify T3 (or T7) primer, ideally select 12 clones

10. library prep: to Nextera XT DNA

- quantify cDNA with Qubit HS dsDNA assay after diluting to o.2ng/µL (critical for proper tagmentation)

- after libraries are done, quantify on Qubit with HS dsDNA assay, and run another PCR with IS primers
- run product on gel, guesstimate the average bp-size (there will be a smear)
- calculate molarity with average bp-sizes and Qubit quantifications, as per Illumina's instructions

- bead-based normalisation for libraries >10nM
- manual normalisation for libraries <10nM