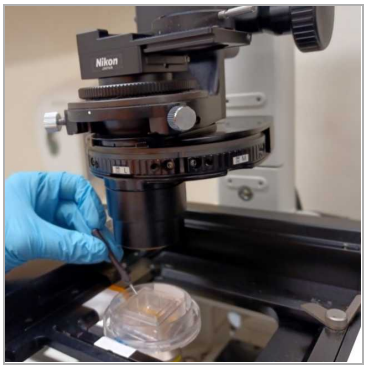

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Manual isolation of nuclei from human brain using CellRaft device and single nucleus Whole Genome Amplification

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ABSTRACT

Protocol for manual nuclear isolation from human brain tissue using Cell Raft device for single cell Whole Genome Amplification.

ATTACHMENTS

Main Protocol.docx | dMDA version 2.pdf | CytoSort Array.pdf | CellRaft.pdf | PTA.pdf

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

Created: Aug 11, 2022

Last Modified: Jun 02, 2023

PROTOCOL integer ID: 68508

Keywords: Single cell, Whole Genome Amplification, Cell Raft, Somatic mutation, ASAPCRN

GUIDELINES

Critical note: Unless otherwise indicated, all the reagents must be kept at 4ºC and all the steps in the protocol must be performed at 4ºC. To preserve nuclear integrity, all the solutions are supplemented with complete cOmplete EDTA-free Protease Inhibitor Cocktail.

References:

- Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. Development. 1992;116(1):201-211. doi:10.1242/dev.116.1.201.
- Wolf HK, Buslei R, Schmidt-Kastner R, et al. NeuN: a useful neuronal marker for diagnostic histopathology. J Histochem Cytochem. 1996;44(10):1167-1171. doi:10.1177/44.10.8813082.
- Cannon JR, Greenamyre JT. NeuN is not a reliable marker of dopamine neurons in rat substantia nigra. Neurosci Lett. 2009;464(1):14-17.
- Reed PJ, Wang M, Erwin JA et al (2017) Single-Cell Whole Genome Amplification and Sequencing to Study Neuronal Mosaicism and Diversity. In: Frade J., Gage F. (eds) Genomic Mosaicism in Neurons and Other Cell Types. Neuromethods, vol 131. Humana Press, New York, NY.

MATERIALS

Commercial Reagents:

	A	B	C	D	E
	Item	Supplier	Catalogue Number	Preparation prior use	Storage
	UltraPure DNase/RNase-Free Distilled Water	Thermo Fisher Scientific	10977049	Aliquot	RT
	PBS(Phosphate Buffered Saline) 10X Solution (pH 7.4)	Fisher Scientific	15815418	Make 1x with dH2O	Fridge
	50x cOmplete Protease Inhibitor Cocktail EDTA-free	Roche via Sigma Aldrich	4693159001	Use 1 tablet in 1 ml dH2O	Freezer (20oC)
	Triton-X100	Sigma Aldrich	T9287	Prepare10% aliquot	RT
	ODGM(Optiprep Density Gradient Medium)	Sigma Aldrich	D1556	Aliquot	Fridge
	Goat Serum	Sigma Aldrich	G9023	Aliquot	Freezer (20oC)
	Cell-Tak™ Cell and Tissu Adhesive	Corning via Sigma Aldrich	DLW354242	No	Fridge

	A	B	C	D	E
	DNase I Solution (1 unit/µL), RNase-free	Thermo Fisher Scientific	89836	Dilute to DNase solution, 200 U/mL and Aliquot	Freezer (20oC)
	TE Buffer (low EDTA)	Thermo Fisher Scientific	12090-015	Aliquot	RT

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #10977049**

PBS (Phosphate Buffered Saline) 10X Solution (pH 7.4) **Fisher Scientific Catalog #15815418**

cOmplete mini EDTA free protease inhibitor cocktail **Merck MilliporeSigma (Sigma-Aldrich) Catalog #4693159001**

,

OptiPrep™ Density Gradient Medium **Merck MilliporeSigma (Sigma-Aldrich) Catalog #D1556**

Goat serum **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G9023**

DNase I Solution (1 unit/µL), RNase-free **Thermo Fisher Catalog #89836**

Low TE Buffer **Invitrogen - Thermo Fisher Catalog #12090-015**

Home-Made Reagents:

	A	B	C
	Item	Sterilization Method	Storage
	1 M MgCl2	Autoclave	RT
	1 M Tris/HCl pH 8.8	Autoclave	RT
	1 M Sucrose	Filtration	Freezer (-20oC)
	1 M KCl	Autoclave	RT
	1 mg/ml DAPI (4',6-diamidino-2-phenylindole)	No	Freezer (-20oC)
	10% Presept	No	RT
	0.1 M NaOH solution	Filtration	Fridge
	2.5 mM NaOH in PBS	Filtration	Fridge
	70% EtOH in dH2O	No	RT

Antibodies for immunodetection

	A	B	C	D	E
	Antibody	Supplier	Catalogue Number	Produced in	Working Concentration
	Sox6 (dopaminergic neuronal nuclei)	Merck	HPA001923	Rabbit	0.5 µg/ml
	Olig2 (oligodendrocyte nuclei)	Abcam	Ab109186	Rabbit	1 µg/ml
	aSyn (alpha-synuclein)	Santa Cruz Biotechnology	sc-12767	Mouse	1 µg/ml
	NeuN clone A60 (cortical neuronal nuclei)*	Millipore	MAB377	Mouse	10 µg/ml
	Secondary anti-mouse IgG conjugated with Alexa (568)	Life Technologies	A21235	Goat	2 µg/ml
	Secondary anti-rabbit IgG conjugated with Alexa (488)	Life Technologies	A11008	Goat	2 µg/ml

Note

***Note:** According to MAB377X description, this antibody works for most neuronal cell types throughout the adult nervous system. However, some neurons fail to be recognized by NeuN at all ages: INL retinal cells, Cajal-Retzius cells, Purkinje cells, inferior olivary and dentate nucleus neurons, and sympathetic ganglion cells and dopaminergic neurons (Mullen et *al.*, 1992; Wolf et *al.*, 1996., Cannon et *al.*, 2009)

aSyn (alpha-synuclein) **Santa Cruz Biotechnology Catalog #sc-12767**

Anti-NeuN Antibody, clone A60 **Merck Millipore (EMD Millipore) Catalog #MAB377**

Specialized consumables:

- CytoSort 200, single, 5-pack - Cell Microsystems - 20200301CS.

General consumables:

- Low binding filtered tips (sterile).

- Low-binding 1.5ml and 0.2 ml tubes (sterile).
- Screwed lid transparent 1.5-2 ml tubes (sterile).
- 1ml syringe without needle (sterile).
- PES Syringe filter, 0.2 µm (sterile).
- Gloves.
- Strong glue e.g., super glue.
- Adhesive tape.

Equipment:

- Tissue culture hood for human sample handling.
- PCR cabinet (Here we used Air Science, Lydiate, UK).
- Dounce tissue grinder set 2 mL (Kimble via Sigma Aldrich - D8938).
- Refrigerated centrifuge for 1.5ml tubes that can achieve at least 13000 x g (here used Sigma Aldrich 1 - 14K Refrigerated Micro Centrifuge).
- Orbital Shaker (Here we used Grant instruments Orbital Shaker PSU-10i).
- Haemocytometer.
- Inverted microscope (Here we used Nikon Eclipse TE300 inverted microscope coupled to a CCD camera - KERN optics).
- CellRaft accessory device (Cell Microsystems, Durham, US), with single reservoir CytoSort array (10,000 rafts).
- Stereoscope.
- General lab pipettes.
- Pair of forceps and scissors.

SAFETY WARNINGS

Please follow the Safety Data Sheets (SDS) for all reagents for safe handling and safety hazards.

This procedure was adapted from:

• Wierman MB, Burbulis IE, Chronister WD, Bekiranov S, MJ MC (2017) Single cell CNV detection in neuronal nuclei. In: Springer (ed) Genomic Mosaicism in Neurons and Cell Types (editors: Frade JM, Gage FH). New York, USA: Humana Press, Springer Nature; ISBN 978-1-4939-7279-1.
• Perez-Rodriguez D, Kalyva M, Leija-Salazar M, et al. Investigation of somatic CNVs in brains of synucleinopathy cases using targeted SNCA analysis and single cell sequencing. Acta Neuropathol Commun. 2019;7(1):219. Published 2019 Dec 23.
• Perez-Rodriguez D, Kalyva M, Santucci C, Proukakis C (2022) Somatic CNV Detection by Single-Cell Whole-Genome Sequencing in Postmortem Human Brain. In: Methods in Molecular Biology: Alzheimer's disease. Vol. 2561, Jerold Chun (Ed).

BEFORE START INSTRUCTIONS

Section 1: Nuclear extraction from human brain

Before starting:

1. Clean Human Tissue handling hood with 0.2 M NaOH, 10% Presept, 70% EtOH, and dH2O.
2. Clean Dounce tissue grinders (prior to use, between samples, and at the end of the experiment) with 0.2 M NaOH, 10% Presept, 70% EtOH, dH2O, and let them dry.

3. UV sterilize and clean PCR hood, centrifuge, pipettes, etc. with 10% Presept, 70% EtOH, and dH2O.
4. Precool centrifuge @4ºC.
5. On the day of the experiment prepare the following Buffers and keep themon ice:

- NIM (Nuclear Isolation Media): 25 mM KCl, 5 mM MgCl2, 10 mM Tris-HCl pH 8.8, 250 mM sucrose, 1 mM dithiothreitol (DTT), 1x cOmplete EDTA-free Protease Inhibitor Cocktail.
- ODN (Optiprep Diluent for Nuclei): 150 mM KCl, 30 mM MgCl2, 60 mM Tris-HCl pH 8.8, 250 mM sucrose, 1x cOmplete EDTA-free Protease Inhibitor Cocktail.
- 25% iodixanol solution (NIM:ODGN:ODN 6:5:1).
- 29% iodixanol solution (ODGN: ODN 29:31).
- NSB (Nuclei Storage Buffer) - if needed: 5 mM MgCl2, 50 mM Tris-HCl (pH 8.8), 166 mM sucrose and 1 mM dithiothreitol (DTT), 1x cOmplete EDTA-free Protease Inhibitor Cocktail.

C. Proceed to Immunodetection of inclusions and/or cell type marker staining

Before starting:

- Prepare PBS: PBS 1x. Keep on ice until use.
- Prepare PBS/PIC: PBS 1x supplemented with 1x cOmplete EDTA-free Protease Inhibitor Cocktail. Keep on ice until use.
- Prepare Blocking Buffer: PBS+PIC supplemented with 10% goat serum. Keep on ice until use.

Section 3. View CytoSort Arrays under the microscope, capture & transfer rafts with single nuclei in tubes for single cell Whole Genome Amplification

Before starting:

- Defreeze an aliquot of diluted DNase I Solution and keep itat RT.
- Prepare aliquots of 50 µl of 70% EtOH and 50 µl of dH2O in 1.5ml tubes in a PCR hood and keep at RT until use. You will need at least 1 aliquot of 70% EtOH and dH2O for each sample you want to amplify. As an example, if you aim to amplify the genome of 10 single nuclei, prepare 15 aliquots of 50 µl of 70% EtOH and 50 µl of dH2O.
- Prepare 0.2ml tubes with buffer depending on the downstream single cell Whole Genome Amplification (scWGA) method: (estimate double the number of tubes than the single nuclei to be collected):

a. Multiple Displacement Amplification (MDA) in droplets (dMDA, Samplix): 2.8µl Lysis Bf (200 mM KOH, 5 mM EDTA pH 8 and 40 mM 1.4 DTT) diluted in 2 µl dH2O

b. PicoPlex (Takara): 5µl TE/Cell Extraction Buffer (depending on the version of the PicoPlex protocol used)

c. Primary Template-directed Amplification/Resolve DNA (PTA, BioSkryB): 3µl Cell Buffer (BioSkryB)

- Mount the CellRaft® Release Device onto a 4× or 10× objective of an inverted microscope and prepare the CytoSort Array for observation under the microscope. For details on this process please see the attached protocols (Attach CytoSort Array and CellRaft protocols):
    1. Cell Microsystems: CellRaft System for Inverted Microscopes (user manual)
    2. Cell Microsystems: CytoSort Array (user manual).

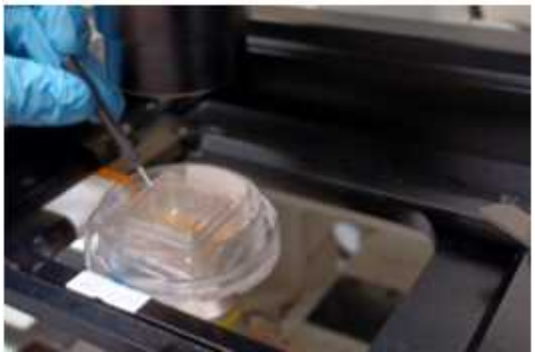

Figure 1. A CytoSort Array is attached to the microscope to allow observation of the rafts and manual picking using a magnetic wand.

Nuclear extraction from human brain:

28m

1 Transfer 20 mg - 50 mg frozen brain tissue to microcentrifuge tube containing 500 µL of NIM. Transfer the tissue in a tube with the lid on using a pair of forceps.

2 **Optional step:** Mincing the tissue in small fragments over a glass plate On ice using a scalpel could significantly decrease the time needed for homogenization and facilitate the trituration with the pipette tips.

3 Gently triturate the tissue with a cut pipette tip (1 mL tip). Repeat several times with pipette tips cut less (estimate 3-4 different cut sizes).

Note

Tissue will be perfectly dissociated when able to pass through an uncut pipette tip.

4 Measure the total volume using 1 mL pipette.

5 Add Triton X-100 solution to the sample to achieve final concentration 0.1% (stock solution 10%).

6 Homogenize using 2 mL Dounce tissue grinders:
a. Grinder A (loose, large clearance pestles): 2-3 strokes.
b. Grinder B (tight, small clearance pestles): 8-12 strokes.

Note

Note: The sample solution should appear homogeneous at this point, but the use of the Dounce grinders allow nuclei to remain intact.

7 Transfer the homogenate to a fresh 1.5 mL microcentrifuge tube.

8 Centrifuge at 1000 x g, 00:08:00, at 4 °C - Discard Supernatant. 8m

9 Resuspend the pellet gently in 700 µL 25% Iodixanol Solution.

10 Layer the sample (avoiding mixing the phases) onto 700 µL 29% iodixanol solution. Use 1 mL syringe without needle to slowly load and suspend the sample on top. As a result, 2 clearly defined phases should appear as follows:

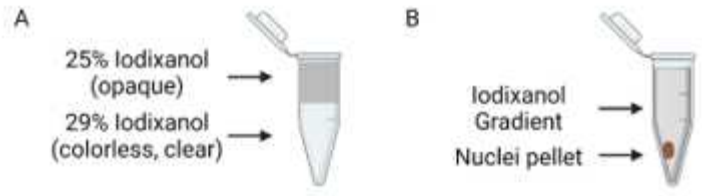

Figure 2: Nuclear isolation using iodixanol gradient. A. Gradient generation solution is generated from 25% iodixanol gradient containing the cell lysate on the top and 29% iodixanol gradient solution on the bottom. B. After centrifugation, the nuclear fraction is near the bottom of the tube.

11 20m

Spin at 10300 x g, 00:20:00, at 4 °C - remove the supernatant leaving 50 µL in the bottom of the ultracentrifuge tube (contain the nuclear fraction).

12 At this point, follow any of the below routes:

Step 12 includes a Step case.

Store nuclear suspensions @4ºC

Proceed to DAPI staining

Proceed to Immunodetection

step case

Store nuclear suspensions @4ºC

Section 1, step 12, Option A

13

Note

Note: Although we routinely use the nuclear suspensions on the day of preparation, others have reported those to be stable at at 4 °C for several weeks when stored in nuclei storage buffer (Reed *et al.*, 2017).

Estimate nuclei number under the microscope:

14 Use a haemocytometer to estimate the number of nuclei in the final suspension. In a good nuclear fraction, most of the nuclei should be seen as single, with few clumps and/or low

amount of cellular/tissue debris.

Manual isolation of single nuclei using a device fixed to an ...

15

Note

Critical note: If possible, do sub-sections 1 and 2 in a PCR cabinet to minimize contamination.

Manual isolation of single nuclei using a device fixed to an ...

16 Visually inspect the CytoSort array prior opening. The pre-sterilized arrays are expected to have a red sticker on packaging. Remove the array from the sealed pouch.

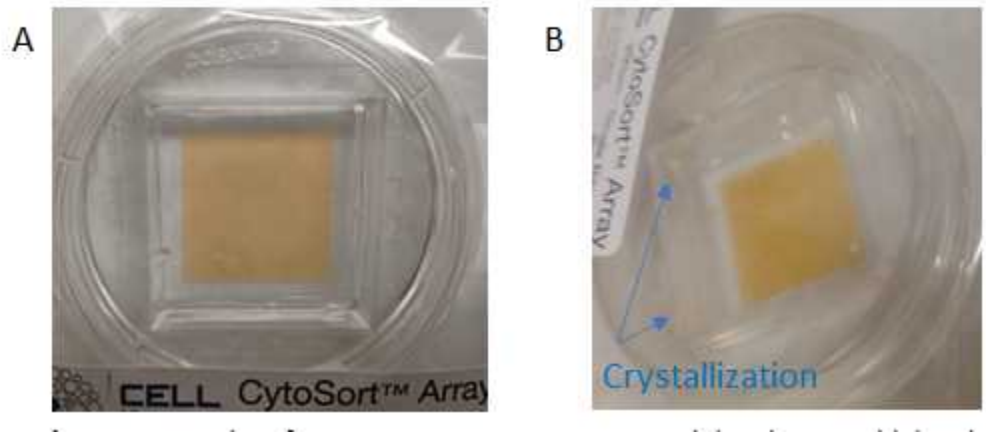

Figure 3. Examples of CytoSort arrays prior to opening (A) without, and (B) with crystallization.

17 **Optional step:** It may be possible to observe crystallization of excess glucose that was used to seal the CytoSort array from exposed air (Figure 2B). In that case:

17.1 Wash the array with 2 mL (pre-warmed at 37 °C) PBS 1x – incubate for 00:03:00. 3m

17.2 Repeat above step 3 times in total: 3m

Wash the array with 2 mL (pre-warmed at 37 °C) PBS 1x – incubate for 00:03:00 (1/3).

17.3 Wash the array with 2 mL (pre-warmed at 37 °C) PBS 1x – incubate for 00:03:00 (2/3). 3m

17.4 Wash the array with 2 mL (pre-warmed at 37 °C) PBS 1x – incubate for 00:03:00 (3/3). 3m

17.5 Cool the array at Room temperature.

17.6 Continue to the next steps.

18 Wash with 2 mL dH2O – incubate for 00:03:00 at Room temperature – Remove water. 3m

19 Repeat the washing with 2 mL dH2O – incubate for 00:03:00 at Room temperature - Remove water. 3m

20 Apply 1 mL of 15 ug/mL Cell-Tak in PBS with [M] 2.5 millimolar (mM) NaOH – incubate for 00:20:00 @ Room temperature – Remove Cell-Talk. 20m

21 Add 2 mL sterile PBS 1x - Incubate for 00:03:00 at Room temperature – Remove PBS. 3m

22 Repeat step 21 (3 times in total):

22.1 Add 2 mL sterile PBS 1x - Incubate for 00:03:00 at Room temperature – Remove PBS (1/3). 3m

22.2 Add 2 mL sterile PBS 1x - Incubate for 00:03:00 at Room temperature – Remove PBS (2/3). 3m

22.3 Add 2 mL sterile PBS 1x - Incubate for 00:03:00 at Room temperature – Remove PBS (3/3). 3m

Manual isolation of single nuclei using a device fixed to an ...

23

Optional step: Insert the CellRaft seeding insert to increase the overall number of single cells available for isolation.

24 Dilute approximately 5000 nuclei in 2 mL of PBS, add pipette them dropwise in the centre of the CytoSort Array.

Note

Critical note: Nuclei should be counted in a haemocytometer prior to seeding. The number of nuclei seeded on the array is a key factor, as too many will lead to few CellRafts with single nuclei, while too little will lead to most of the CellRafts being empty, according to a Poisson distribution. Thus, seeding the array with a nuclei: wells ratio 1:2 or 1:3 increases the proportion of wells capturing a single nucleus *versus* wells capturing none or >2 nuclei.

25 Move the CytoSort Array carefully to ensure the nuclei settle evenly within the array (like seeding cells for cell culture).

26 Allow it to settle for 02:00:00 - O/N in the fridge. 2h

Note

Note 1: Use parafilm, paper towel and foil to properly seal the array to prevent desiccation
Note 2: From our experience, nuclei are stable on the array for up to 336:00:00 when stored properly sealed in the fridge.

Isolation of rafts containing the nuclei of interest:

20m

27 View CytoSort Arrays under the 10x magnification objective with appropriate fluorescence filters, to select rafts (microwells) containing a single nucleus with the desired characteristics and take photos (if needed Fig 4).

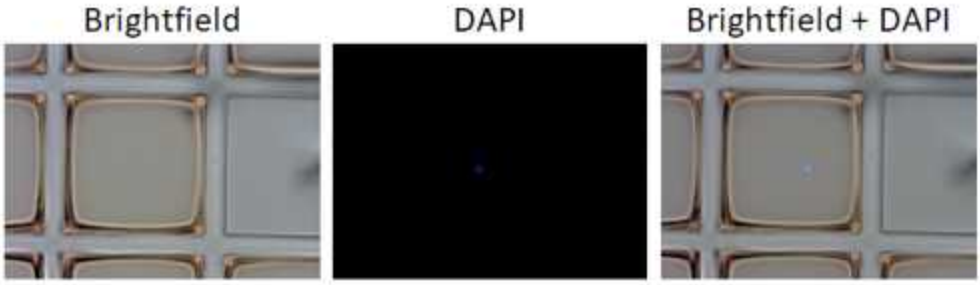

Figure 4. Manual picking of rafts containing the nuclei of interest. A CellRaft with a single nucleus stained by DAPI.

28 Individually release the arrays of interest (Figure 5).

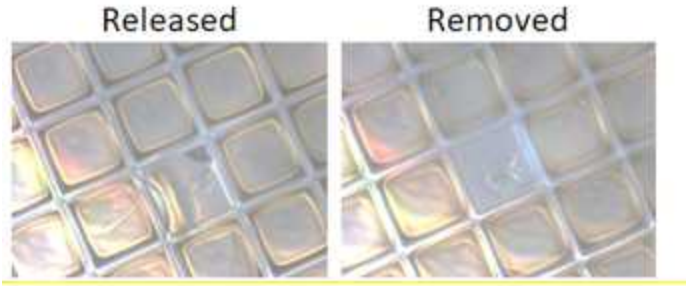

Figure 5. Example of released and removed CellRafts.

29 Place the magnetic wand in the tubes containing DNase-I diluted solution.

Note

Critical note: DNAse-I solution is used to reduce cross-contamination between samples. This may require optimisation in each lab.

30 Wash the wand with 70% EtOH and then with dH2O.

31 Capture, release and remove the released raft to the already prepared 0.2 mL tubes with the buffer needed for scWGA using the magnetic want. Then, centrifuge the tubes briefly.

32 When performing CellRaft isolation, it is highly recommended to ensure the rafts are properly released at the bottom of the tube and not in the tube wall, as they would desiccate. To do so, visually inspect every tube after collection under a stereoscopic microscope; rafts will appear as small orange squares.

Note

Critical note: As controls, use at least one of each of the following per experiment:

- Negative control 1: a tube with no raft, in which the magnetic wand has been dipped after it was dipped into the array covering fluid.
- Negative control 2: a raft with no nucleus.
- Positive control: 15 pg of genomic DNA.

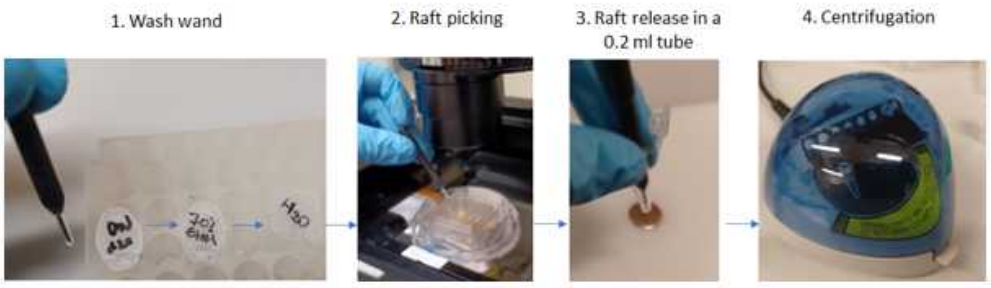

Figure 6. Wand washing, raft picking and release in 0.2 ml tubes.

33 Freeze the samples at -80 °C or proceed directly to scWGA. 20m

For scWGA we have used the following methodologies:

- PicoPlex (R300670, R300672 and R300722) according to Takara’s protocols.
- dMDA with heat denaturation and Buffer Generation (dMDA protocol attached).
- PTA (PTA protocol attached) but for step 7: incubate for 00:20:00 at Room temperature.

Note

Note: So far, we have successfully used the PicoPlex method with and without nuclei immunostaining. However, we have not tested the dMDA and PTA methods in combination with immunostaining, but we have just stained the nuclei with DAPI staining.