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Endosomal, lysosomal, mitochondrial, or Golgi immunoprecipitation for quantitative proteomics

Forked from Endosomal and lysosomal immunoprecipitation for proteomics, lipidomics, and TEM

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We use this protocol and it's working

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Disclaimer

Abstract

Previous studies have developed methods for the immunoisolation of lysosomes, mitochondria, EEA1-positive endosomes, Golgi, and other organelles from non-denaturing / non-detergent extracts. Here we describe our most up-to-date protocols for these approaches. Specifically, we describe methods to isolate lysosomes, mitochondria, EEA1-positive endosomes, Golgi, and the preparation of peptides for proteomics applications. We structure our protocol with a modular framework for the future addition of new cell types and organelle-IPs by anyone in the community.

Materials

Buffers (see table below for chemicals)
PBS/DPBS: 137 mM NaCl , 2.7 mM KCl, 10 mM Phosphate, pH 7.4 (MedChemExpress HY-K1023)

KPBS Buffer (also called Mito-KPBS, Golgi-KPBS, and Lyso-KPBS): 136mM KCL, 10 mM KH2PO4. Adjust to pH 7.25 with KOH. (Note On the day of use, add Roche cOmplete protease inhibitor cocktail tablet (REF# 11873580001) and Roche PhosSTOP tablet (REF# 04906837001)
→ To make 500 mL: Dissolve 5.07 g KCl and 0.68 g KH_2PO_4 in 450 mL water. Adjust to pH 7.25 with KOH and then bring up the volume to 500 mL.

High-Salt-KPBS (For LysoIP): KPBS + 150 mM NaCl

EndoIP-KPBS (For EndoIP): 25 mM KCl, 100 mM potassium phosphate, pH 7.2
→ To make 500 mL, dissolve 0.93 g KCl, 5.366 g K_2HPO_4, 2.61 g KH_2PO_4 in 450 mL water, adjust to pH 7.2 with KOH, then top off volume with water.

Antibodies and chemicals:

	A	B	C	D	E	F
	REAGENT or RESOURCE	**SOURCE**	**IDENTIFIER**	**ORGANELLE**	**WB dilution**	**IF dilution**
	Antibodies					
	anti-EEA1 (C45B10) rabbit mAb	Cell Signaling Technology	3288	Early/sorting endosome	1:1000	1:200
	anti-RAB5 (C8B1) rabbit mAb	Cell Signaling Technology	3547	Early endosome	1:1000	1:200
	anti-PSEN1 (D39D1) rabbit mAb	Cell Signaling Technology	5643	ER/endosome/Golgi	1:1000	
	anti-PSEN2/AD5 (EP1515Y) rabbit mAb	Abcam	ab51249	ER/Golgi/endosome	1:1000	
	anti-LAMP1 (D2D11) rabbit mAb	Cell Signaling Technology	9091	Lysosome/endosome	1:1000	1:200
	anti-LAMP1 (D4O1S) Mouse mAb	Cell Signaling Technology	15665	Lysosome/endosome	-	1:200 (better)
	anti-LAMP2 (D5C2P) rabbit mAb	Cell Signaling Technology	49067	Lysosome	1:1000	
	anti-TMEM192 rabbit pAb	Proteintech	28263-1-AP	Lysosome	1:1000	
	anti-HA (6E2) mouse mAb	Cell Signaling Technology	2367	LysoTag, GolgiTag, or MitoTag	1:1000	

	A	B	C	D	E	F
	anti-HA (3F10) rat mAb	Sigma	1186742300	LysoTag, GolgiTag, or MitoTag	-	1:400
	anti-FLAG M2 mouse mAb	Sigma-Aldrich	F1804	Epitope (EndoTag)	1:1000	-
	anti-DYKDDDK mouse mAb	Thermo	MA1-91878	Epitope (EndoTag)	-	1:100
	anti-ZO-1 rabbit pAb	Proteintech	21773-1-AP	Plasma membrane	1:1000	
	anti-Giantin (GOLGB1)	Abcam	ab37266	Golgi	1:1000	1:100
	anti-YIPF4	Proteintech	15473-1-AP	Golgi	1:1000	1:200
	anti-Golga1 rabbit pAb	Proteintech	12640-1-AP	Golgi	1:1000	
	anti-TMEM115	Novus	80898	Golgi	1:500	1:100
	anti-GOLGA2/GM130 rabbit pAB	Proteintech	11308-1-AP	Golgi	1:1000	1:400
	anti-Golgin97 (CDF4) mouse mAb	Thermo	# A-21270	Golgi	1:1000	1:50
	anti-Calreticulin rabbit pAb	Proteintech	10292-1-AP	Endoplasmic reticulum	1:1000	
	anti-S6K rabbit pAb	Proteintech	14485-1-AP	Ribosome (cytoplasm)	1:1000	
	anti-RAB11 (D4F5) rabbit mAb	Cell Signaling Technology	5589	Recycling endosome	1:500	
	anti-Lamin A/C (4C11) mouse mAb	Cell Signaling Technology	4777	Nucleus	1:1000	
	anti-VDAC1/Porin rabbit pAb	Proteintech	55259-1-AP	Mitochondrion	1:1000	
	anti-RAB7 (D95F2) rabbit mAb	Proteintech	9367	Late endosome/lysosome	1:1000	
	anti-VPS35	Santa Cruz	374372	Endosome	1:1000	1:200
	anti-GAPDH (D16H11) XP rabbit mAb	Cell Signaling Technology	5174	Loading control	1:1000	
	anti-PEX19 rabbit pAb	Proteintech	14713-1-AP	Peroxosome	1:1000	
	anti-CD71/TFR1 (D7G9X) rabbit mAb	Cell Signaling Technology	13113	Endosome/ plasma membrane	1:1000	

	A	B	C	D	E	F
	anti-HSP90 (3F11C1) mouse mAb	Proteintech	60318-1-Ig	Cytoplasm	1:1000	
	anti-HSP60 (LK-1) mouse mAb	Abcam	ab59467	Mitochondria		1:400
	IRDye 680RD Goat anti-Rabbit IgG secondary antibody	Li-Cor	926-68071	-	1:10,000	-
	IRDye 680RD Goat anti-Mouse IgG secondary antibody	Li-Cor	926-68070	-	1:10,000	-
	IRDye 800CW Goat anti-Rabbit IgG secondary antibody	Li-Cor	926-32211	-	1:10,000	-
	IRDye 800CW Goat anti-Mouse IgG secondary antibody	Li-Cor	926-32210	-	1:10,000	-
	Goat anti-Rabbit IgG, HRP-linked antibody	Cell Signaling Technology	7474P2	-	1:10,000	-
	Goat anti-Rabbit IgG HRP conjugate	Bio-Rad	1706515	-	1:10,000	-
	Goat anti-Mouse IgG HRP conjugate	Bio-Rad	1706516	-	1:10,000	-
	Anti-mouse 488	Thermo	A-11029	-	-	1:200
	Anti-mouse 568	Thermo	A-11029	-	-	1:200
	Anti-mouse 647	Thermo	A-11029	-	-	1:200
	Anti-rat 488	Thermo	A-11029	-	-	1:200
	Anti-rat 647	Thermo	A-11029	-	-	1:200
	Anti-Rabbit 488	Thermo	A-11034	-	-	1:200
	Anti-Rabbit 568	Thermo	A-11029	-	-	1:200
	Anti-Rabbit 647	Thermo	A-11029	-	-	1:200
	Chemicals, peptides, and recombinant proteins					
	anti-FLAG M2 magnetic beads	Sigma-Aldrich	M8823			
	Pierce anti-HA magnetic beads	Thermo Fisher Scientific	88837			

	A	B	C	D	E	F
	TMTProTM 18Plex Label Reagent set	Thermo Fisher Scientific	A52045			
	Pierce™ High pH Reversed-Phase Peptide Fractionation Kit	Thermo Fisher Scientific	84868			
	HyClone Fetal bovine serum	GE Healthcare	SB30910			
	Puromycin	Sigma-Aldrich	P9620			
	G418 (Geneticin)	Invivogen	ant-gn-2			
	Dulbecco's MEM (DMEM), high glucose, pyruvate	GIBCO / Invitrogen	11995			
	Penicillin-Streptomycin (10,000 U/mL)	Thermo	15140163			
	GlutaMAX™ Supplement	Thermo	35050061			
	RPMI	Thermo	11875119			
	PhosSTOP	Roche	04906845001			
	Complete EDTA-free protease inhibitor cocktail	Sigma-Aldrich	11873580001			
	Tris(2-carboxyethyl)phosphine hydrochloride solution	Sigma-Aldrich	646547			
	Iodoacetamide	Sigma-Aldrich	I1149			
	Trichloroacetic acid solution 6.1 N (TCA)	Sigma-Aldrich	T0699			
	Trifluoroacetic acid (TFA)	fisher scientific	A11650			
	Acetonitrile (ACN)	Sigma	34851-4X4L			
	HPLC Water	Sigma	270733			
	Hydroxylamine solution 50 wt. %	Sigma-Aldrich	438227			
	Methanol	Sigma	34860-2L-R			
	Ethanol	VWR	TX89125172HU			

	A	B	C	D	E	F
	Formic Acid (FA)	Sigma-Aldrich	5330020050			
	Pierce Trypsin Protease, MS grade	Thermo Fisher Scientific	90305			
	Lysyl endopeptidaseR (Lys-C)	Wako	129-02541			
	REVERT 700 total protein stain kit	Li-Cor	926-11016			
	NuPAGE LDS sample buffer (4X)	Thermo Fisher Scientific	NP0007			
	NuPAGE sample reducing agent (10X)	Thermo Fisher Scientific	NP0009			
	NuPAGE MES SDS Running Buffer (20X)	Thermo Fisher Scientific	NP0002			
	Immobilon-FL PVDF Membrane	Millipore	IPFL00010			
	WHEATON Dounce Tissue Grinder, 7 mL	DWK Life Sciences	357542			
	KIMBLE KONTES Dounce Tissue Grinder, 2 mL	DWK Life Sciences	885300-0002			
	Nonidet P40 substitute	Sigma-Aldrich	74385			
	Urea	Sigma-Aldrich	U5378			
	EPPS 0.2M buffer solution, pH 8.5	Alfa Aesar	J61476.AE			
	Empore C18 47 mm Extraction Disc, Model 2215	3M	98060402173			
	Sep-Pak C18 1 cc Vac Cartridge	Waters	WAT054955			
	RIPA lysis and extraction buffer	Thermo Fisher Scientific	89900			

	A	B	C	D	E	F
	Experimental models: Cell lines					
	293 cells	ATCC	CRL-1573			
	293T cells	ATCC	CRL-3216			
	THP-1 cells	ATCC	TIB-202			

Troubleshooting

Before start

Note that a significant amount of information is contained in the "Materials" section.

Construct selection, cell line engineering, and validation

1 For LysoIP: Express the LysoTag, TMEM192-3xHA, endogenously (Addgene#175777) or with stable lentiviral overexpression (e.g., with either Addgene#102930 or Addgene#134631). Keep the parental cell line as a negative control.

For EndoIP: Express the EndoTag, 3xFLAG-EEA1, endogenously (Addgene#214989 or Addgene#214990) or with stable lentiviral overexpression (e.g., with either Addgene#176491, Addgene#214994, Addgene#214995). Keep the parental cell line as a negative control.

For MitoIP: Express the MitoTag, 3xHA-OMP25, with stable lentiviral overexpression (e.g., with Addgene#83356). Keep the parental cell line, or a different epitope tag (e.g., Addgene#83355) as a negative control.

For GolgiIP: Express the GolgiTag, TMEM115-3xHA, endogenously (Addgene#214998, Addgene#214999, or Addgene#215000) or with stable lentiviral overexpression (e.g., with either Addgene#214996, Addgene#214997). Keep the parental cell line as a negative control.

Note

For stable overexpression with lentivirus, we recommend FACS sorting the cells for medium/low expressors to preserve organellar integrity/identity and localization of the IP handles. Antibiotic selection after introducing a transgene with lentivirus can lead to orders of magnitude differences in expression levels between cells. Check that the cells do not lose the transgene during scale-up (this has not been a problem for us when FACS sorting cells with "purity" or "ultra purity" mode enabled).

We recommend checking gentle lysis feasibility for a given cell type prior to incorporating these tags and further validation/optimization.

2 Validate tag successful tag incorporation by western blot.

For LysoIP, blot for the HA tag (1:1000, CST #2367S; AB_10691311) and endogenous TMEM192 (1:1000, Proteintech 28263-1-AP, AB_2881099).

For MitoIP, blot for the HA tag (1:1000, CST #2367S; AB_10691311).

For GolgiIP, blot for the HA tag (1:1000, CST #2367S; AB_10691311) and endogenous TMEM115 (1:500, Novus NBP1-80898; AB_11002107). Note that this antibody is fairly

nonspecific (several nonspecific bands are more prominent than TMEM115 itself) and the reactivity of this antibody is untested in mouse cells.

For EndoIP, blot for the FLAG tag (1:1000, Sigma F1804; AB_262044) and endogenous EEA1 (1:1000, CST #3288S; AB_2096811).

3 Validate tag successful tag incorporation by immunoflourescence. See below (3.1) for a protocol link.

For LysoIP from human cells, check colocalization between the HA tag (1:400, Sigma 11867423001; AB_390918) and LAMP1 (1:200, CST # 9091; AB_2687579 or 1:200 CST #15665; AB_2798750). While PFA-TritonX fixation-permeabilization works, PFA-methanol resolves lysosomes better.

For MitoIP from human or mouse cells, check colocalization between the HA tag (1:400, Sigma 11867423001; AB_390918) and HSP60 (1:400, Abcam ab59457; AB_2121285). PFA-TritonX fixation-permeabilization works fine.

For GolgiIP from human cells, check colocalization between the HA tag (1:400 Sigma 11867423001; AB_390918) and GOLGA2 (Proteintech 11308-1-AP; AB_2115327). From mouse cells, check colocalization between the HA tag (1:400 Sigma 11867423001; AB_390918) and YIPF4 (1:200 Proteintech 15473-1-AP; AB_2217206). PFA-TritonX fixation-permeabilization works fine.

For EndoIP, check colocalization between the FLAG tag (1:100, Thermo MA1-91878; AB_1957945) and VPS35 (1:200, Santa Cruz sc-374372; AB_10988942) or RAB5 (1:200, Cell signalling #3547; RRID:AB_2300649). PFA-TritonX fixation-permeabilization works fine. When overexpressed using a lentivirus, EndoTag localization can be heterogeneous across cells (e.g., the EndoTag might be cytosolic in up to 50% of cells, even with a clonal population). We've found that organelle isolation from this mixed population still works, although you are likely losing material.

Note

For each organelleIP handle, ensure that the tag localization does not change in response to treatment, genetic perturbation, etc., so that the identities of the isolated organelles are the same between control and experimental condition(s).

3.1 We often use the following protocol for IF:
dx.doi.org/10.17504/protocols.io.kxygxyeeol8j/v1

Note

See our pubpub publication for representative confocal images.

4 Proceed to plate and harvest cells ("cell plating and harvesting", perform the appropriate OrganelleIP protocol ("OrganelleIP"), then prep peptides for proteomics ("proteomics prep", or stop with a western blot).

STEP CASE

OrganelleIP 16 steps

5 The following steps are largely the same for Lyso, Mito, Golgi, and EndoIPs. Please be aware, however, that the buffer and magnetic beads are different for EndoIP, and that LysoIP contains a high salt wash different from Mito/Golgi IPs. We also recommend different incubation times for the different organelle isolations; however, folks in our lab have been successful in enriching organelles with a range of incubation times (e.g., 10 - 50 mins). Smaller bead binding times lead to more specific isolation, at the cost of less material.

Note

Include a set of negative controls (a cell line without the appropriate epitope tag) within every organelle-IP experiment. It's otherwise very difficult to tell whether proteins that appear "significantly changing" in response to a treatment, genotype, etc. do so because a difference in the background, rather than a real localization change. We discuss how to use this control to check organelle enrichment at the end of the "Proteomics prep" section.

Cells cannot be harvested and then frozen at any step for these protocols--the first pause point is after elution from the beads.

STEP CASE

LysoIP 15 steps

Lysosomal immunoprecipitation (Lyso-IP) for organellar proteomics

6 Invert **α-HA** magnetic beads several times gently to resuspend them, and then aliquot (60-80 µL of bead slurry per replicate, plus 10% excess) into a low-bind tube. Rotate end-over-end at 4 ºC until use (below).

Note

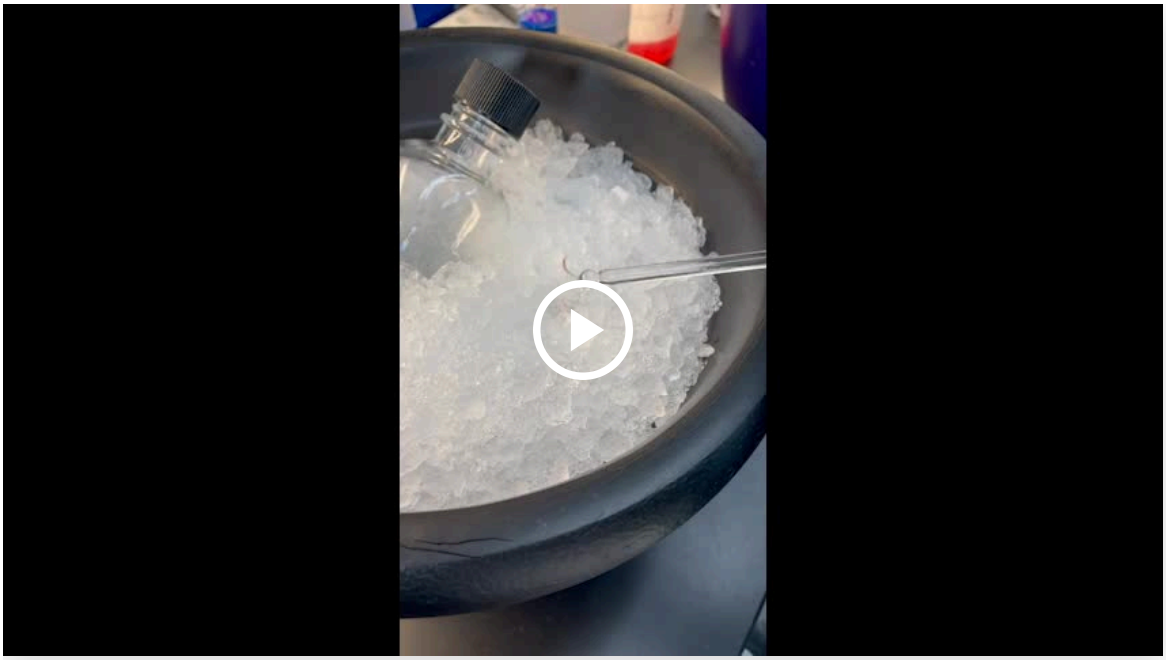
We've successfully used various amounts of beads. More beads (e.g., 80-100 µL slurry per replicate) are ideal for proteomics experiments, but bead volume will quickly increase the cost of the experiment.

7 Proceeding from "cell plating and harvesting": Discard supernatants, wash pellets once with 1 mL of ice-cold Lyso-KPBS buffer (136mM KCL, 10 mM potassium phosphate, pH 7.25, protease and phosphatase inhibitors), and pellet again at 1,000 xg for 2 min at 4 ºC. Work quickly, because the cells will likely start to lyse.

Note

Include phosphatase inhibitors even if downstream analysis only includes proteomics/WB. Peripheral membrane-associated proteins depend on lipid identity.

8 Resuspend cell pellets in 1 mL of Lyso-KBPS buffer supplemented with protease and phosphatase inhibitor tablets and lyse with 25-30 strokes with a 2 mL Dounce homogenizer on ice. Collect with a Pasteur pipette. Harvest into low-bind microcentrifuge tubes.



Note

Dounce homogenization should be optimized prior to scale-up, as it depends heavily on the user and the cell line. We tend towards the upper end (30 strokes) for HEK/HeLa cells, and less (25-30) for differentiated THP-1s. It's likely that more extended cell lines rupture faster than cells with more spherical morphology. One way to check lysis efficiency is to Dounce 1-10 cm plate in 1-mL lysis buffer, dilute an aliquot of the lysate in KPBS, and then stain with trypan blue. We've found that automated counting can be inconsistent, so it's best to also look yourself. Don't expect more than 40-60 % of cells to be lysed, and never attempt to reach 100%.

Importantly, to Dounce, slowly and deliberately stroke the pestle up and down without introducing bubbles. Bring the pestle up such that it doesn't quite leave the liquid level to avoid bubbles.

8.1 Add an additional 200 µL of ice-cold Lyso-KPBS to the homogenizer and collect with a Pasteur pipette. Wash the homogenizer with KPBS (without inhibitors) between replicates, and thoroughly wash between experimental conditions (with a final KPBS rinse).

9 Centrifuge lysed cells at 1,000 xg for 5 min at 4 ºC, and transfer the post-nuclear supernatants (PNS) to new tubes on ice.

Note

From here on out, we use P1000 tips for transfer and washes. While we've found that normal tips work fine, wide-bore tips minimize the chance of organelle damage and are therefore preferable.

9.1 Spin the PNS from step 8 again at 1,000 xg for 5 min at 4 ºC, and transfer the final PNS to new tubes on ice.

10 Transfer 10 µL of each PNS to a new tube and combine with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent for later analysis by Western blot; "INPUT sample". These amounts can be doubled if needed.

Note

Antibodies that work well for proteins that localize to particular organelles are listed in the "Materials" tab.

11 Wash α-HA magnetic beads (60-80 µL of bead slurry per replicate) three times with 1 mL Lyso-KPBS buffer and then resuspend them in Lyso-KPBS with inhibitors using a wide-

bore P1000 pipette. Add the resuspended bead slurry to each PNS, and incubate samples at 4 ºC for **15 min** with gentle rotation.

12 Separate beads from the lysate with a magnetic stand, and collect the flow through ("FLOW THROUGH"). For Western blot analysis, combine 10 µL of each flow through with 20 µL of RIPA lysis buffer and 10 µL of 4X LDS buffer with reducing agent.

13 Using a magnetic stand, wash beads twice with 1 mL of ice-cold **high salt** Lyso-KPBS buffer (136mM KCL, 10 mM potassium phosphate, pH 7.25, **150 mM NaCl**) with inhibitors.

Note

To wash the beads, aspirate the solution, remove the beads from the magnetic rack, and gently resuspend them 2-3 times with a wide bore P1000 pipette before putting them back on the magnetic rack. Work as quickly as possible.

Allow at least 30 s for beads to adhere to the magnet.

14 Wash once with 1 mL ice-cold Lyso-KPBS (136mM KCl, 10 mM potassium phosphate, pH 7.25) with inhibitors. Transfer the samples to new tubes.

Note

Transferring the beads to new tube reduces nonspecific background that binds to the tube during the PNS bead-binding step.

15 Using a magnetic stand, pellet and aspirate the final Lyso-KPBS solution. Elute samples by addition of 90 µL 0.5% NP-40 in KBPS with inhibitors for 30 min at 4 ºC with gentle rotation. Separate beads from the lysate with a magnetic stand, and collect the flow through ("ELUTE").

Note

Alternative elution methods can be used for different downstream applications. Detergent solubilization works well for western blot/MS. Note that the LysoTag will mostly remain associated with the beads.

16 Resuspend beads in an additional 30 µL 0.5% NP-40 in KBPS with inhibitors to harvest any additional protein (collect the flow through). Combine with the eluate from step 14.

17 For Western blot analysis, combine 20 µL of each eluate with 6.7 µL of 4X LDS buffer with reducing agent. Immediately process remainder of eluates or snap freeze in liquid nitrogen and store at -80 ºC until processing for mass spectrometry. For MS processing, see the "Proteomics prep" step case above.

17.1 For a representative Western Blot, see Figure 1C: **https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5704967/**

Note

A negative control IP (or set of IPs) must be included to check enrichment properly.

Protocol references

https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5704967/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC10193996/
https://pubmed.ncbi.nlm.nih.gov/36245040/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5851482/
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5030821/
https://www.biorxiv.org/content/10.1101/2023.12.18.572249v1
dx.doi.org/10.17504/protocols.io.4r3l24kxxg1y/v2
dx.doi.org/10.17504/protocols.io.6qpvrdjrogmk/v2
dx.doi.org/10.17504/protocols.io.bybjpskn