Protocol:	**Early Endosome Fusion Assay**
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Reference:	Ohya et al., Nature 2009

1. Solutions and reagents preparation

Energy:
800 mM Creatine Phosphate in water
4 mg/ml Creatine Kinase in 50% glycerol, 5 mM NaH_2PO_4 pH 7.0
100 mM ATP in water pH 7.0

Fusion Buffer: (1X)
12.5 mM Hepes pH 7.4
1.5 mM MgOAc
3 mM Imidazole
1 mM DTT
75 mM KOAc
- to prepare 50X solution, the pH should be around 8.8 and check that when diluted to 1X it will be 7.4.

Wash buffer (for assay)
100 mM NaCl
50 mM Tris-HCl pH 7.4
2 mg/ml BSA
2% Triton X-100

SIM (for Early endosome prep)
250 mM Sucrose
3 mM Imidazole
1 mM MgCl2
pH 7.4

KEHM (for cytosol prep)
50 mM KCl
50 mM Hepes, pH 7.4
10 mM EGTA
2 mM MgCl2

62% Sucrose (for density gradient)
80.49 g sucrose
3 mM Imidazole
100 ml water

35% Sucrose (for density gradient)
40.3 g sucrose
3 mM Imidazole
100 ml water

25% Sucrose (for density gradient)
27.59% sucrose
3 mM Imidazole
100 ml water

Ruthenium tag of Secondary antibody

Take secondary rabbit-anti-sheep Fc (IgG) usually at 1.7 mg/ml (sodium phosphate/NaCl) from dianova.
TAG-NHS (usually at 1.7-2 mg/ml in DMSO at -20 degrees).
Mix them as outlined below to a 4 or 5 molar excess of TAG to antibody.

antibody (150kDa)	=6.66 nmoles	= 588 µl (1.7 mg/ml)
TAG-NHS (1.057kDa)	=33.3 nmoles	=23.5 µl TAG (if 1.5mg/ml)
		=412 µl PBS-1
		1024 µl

Vortex and incubate with rotation at room temperature for 60 minutes, covered with foil.
Stop reaction by adding 40 µl 2M glycine.
Incubate further 10 minutes at r.t./dark.
Load onto a 2.5 ml PD-10 column equilibrated with PBS-2.
Begin eluting with 15 x 500 µl PBS-2 and collect 500 µl fractions. First fraction will still be void volume.
Measure the protein concentration by biorad of 20 µl/fraction at OD_{595}
Measure the TAG concentration of each total fraction at OD_{455}.
Pool fractions with TAG and calculate the protein concentration (minumum should be 100-200 µg/ml).
Calculate the ratio of TAG/antibody

$$\frac{\text{TAG OD}/13700}{\text{IgG (mol/L)}} = \text{ratio.}$$

PBS-1
0.15M potassium phosphate
0.15M sodium chloride
pH 7.8

PBS-2
0.15M potassium phosphate
0.15M sodium chloride
0.05% sodium azide
pH 7.2

To increase the stability of antibody it is recommended to add bovine serum albumine to the final concentration of 3% (all protein concentration measurements need to be done beforehand).
Store the labeled antibody in aliquots at –20 degrees, freeze-thawing should be avoided.

S-Hela growth for 2.5 Litre Spinner Culture

Solutions and Media

S-MEM, containing:
- L-glutamine
- Non-essential amino acids
- Fetal Calf Serum (5%)
- Penicillin/Streptomycin

Trypsin-EDTA

The Cells.

-Prepare 4 confluent medium sized flasks (175 cm^2) of S-Hela in S-MEM.
-Trypsinize them and add to 400 ml of S-MEM media in a 1L spinner flask. Place on a stirrer in the 37 degree room for 24 hours.
-The next day, transfer these spinning cells into a 3L spinner flask containing 2L of fresh media.
-Grow at 37 degrees until cells are 0.8-1.2 x 10^6 cells/ml. This should take 4 days. If the cells are too dense they will not internalize, so dilute them and grow into log phase again.

The Harvest.

-Collect cells in GS-3 bottles and spin at 3K for 10 minutes at room temperature.
-Wash in PBS, room temp.
-Spin 3K for 10 min.
-Transfer washed pellet into 50 ml falcon tube and wash again with PBS.
-Spin in tabletop fuge 3 K for 10 minutes, r.t.
-If preparing cytosol, resuspend cells in 1 cell volumes of ice cold cytosol buffer (KEHM) containing freshly added 1mM DTT, 1X CLAAP and 1X APMSF, then proceed to "cracking". The half life of most protease inhibitors is within the 10-20 minute range once in water so crack immediately after adding the cocktails.

<u>Acceptor internalization:</u>
-Anti-transferrin antibodies are dialyzed against CO_2-independant media (+ 0.2 % BSA) before-hand and prewarmed just prior to use.
-Resuspend cells in prewarmed internalization media (containing antibodies) to a final volume of 10-15 ml (2-3 cell volumes).
-Incubate (internalize) at 37 degrees in a waterbath for 5 minutes.
-Transfer immediately to an ice water bath and spin out the cells at 4 degrees in the tabletop 3K for 10 minutes.
-Keep the antibody-containing media for re-use (filter sterilize and adjust pH).
-Resuspend the cells in cold homogenization buffer (SIM) and wash 2 times.
-Resuspend final cell pellet in 2 cell volumes of ice cold SIM containing freshly added CLAAP,1mM DTT and APMSF (see above).
-Proceed to "cracking".

<u>Donor internalization:</u>

-Resuspend cell pellet in 10-15 ml prewarmed CO2-independant media + 0.2 % BSA, which contains 20 μg/ml biotinylated transferrin (stock is 10 mg/ml in PBS).
-Incubate 5 minutes at 37 degrees in a waterbath.
-Dilute up to 50 ml with ice cold SIM and transfer to ice bath.
-Spin in tabletop fuge at 3K, 10 minutes and wash 2 times with SIM.
-Resuspend final cell pellet in 2 cell volumes of ice cold SIM containing freshly added CLAAP, 1mM DTT and APMSF.
-Proceed to "cracking".

Cracking

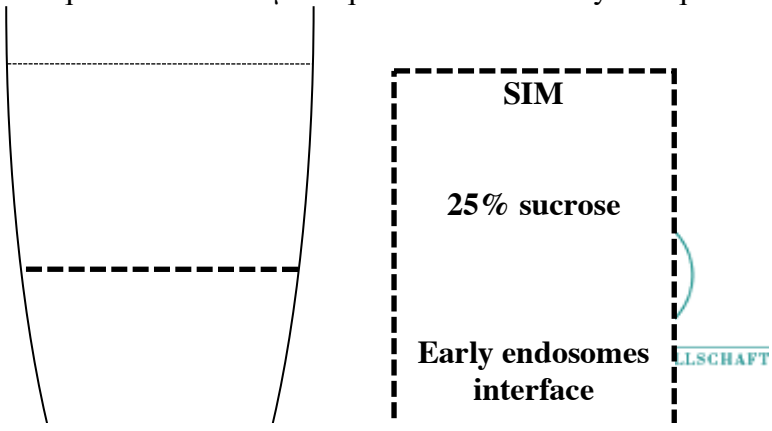
-Assemble the cell cracker (made at the EMBL) using ball bearing size 8.004 and two syringes 5 or 10 mls, depending on the cell volumes. Pre-cool the cracker in the cold room.
-From here on, all steps must be at 4 degrees to avoid protein degradation and keep label at early endosome level.
-Passage the cells through the cracker 6 or 7 times before you check under the microscope to see the breakage. This takes much strength and patience, since there is extreme pressure in the chamber. Wear goggles or glasses in case the syringe pops out and the homogenate flies around.
-Compare the broken cells (10μl on a coverslip) with unbroken. It is probable that you will need another passage or two depending on your strength.
-When all the cells are broken, if preparing cytosol, continue to cytosol protocol. If preparing endosomes, spin the cell homogenate in the tabletop at 4K for 20 minutes at 4 degrees.
-Collect the supernatant (PNS). Sometimes it is difficult to distinguish the interface between the nuclei/unbroken cells and the supernatant so look through the light. If it is a problem, you can dilute the homogenate with SIM and re-spin. The PNS can be quick-frozen at -80 for a later date if you need to, otherwise continue with the sucrose density gradient.

Sucrose Density Gradient

-Adjust the PNS sucrose concentration to 40.6% sucrose using the refractometer and a ice cold 62% sucrose stock. Begin by adding 600 μl of 62% sucrose to 500 μl PNS.
-One pot of 2.4L should be divided among 6 tubes to fill one rotor.
-In the cold room, use a syringe to load this amount at the bottom of a SW40 tube. Carefully cover this with 4 ml of cold 35% sucrose. Overlay this with another 4 ml of 25% sucrose. Fill the tube with SIM.
-Spin the gradients at 35K for 6 hours at 4 degrees.
-Collect the early endosome band at the 25-35% interface using the peristaltic pump and aliquot in 200 μl fractions. Take 40 μl for a biorad protein assay and quick-freeze the rest for storage at -80.

Cytosol

-Take cell homogenate and spin in TLA 100.4 (about 3 ml per tube) at 80K for 30 minutes at 4 degrees.
-Remove the supernatant (cytosol) and aliquot. Take a 10 μl aliquot for biorad assay and quick freeze the rest at -80.



Fusion Assay

Each fusion assay is carried out in 20 µl and contains:
0.4 µl 50X fusion buffer.
1.0 µl cold holotransferrin (40 mg/ml in PBS)
1.3 µl energy mix (freshly mixed 1:1:1 each creatine kinase, creatine phosphate and ATP)
1-2 µl donor endosomes or donor clathrin-coated vesicles
2-4 µl acceptor endosomes
3 mg/ml HeLa cytosol
remainder of 20 microlitres filled with water and/or reagents to be tested.

Basal experiment consists of +/- energy
+/- cytosol
+/- Rab-GDI
100% input (5% total solubilized membranes)

In order to avoiding pipetting mistakes it is advisable to prepare a premix of reagents present in every reaction such as fusion buffer, transferrin, donor and acceptor membranes, as well as energy mix and cytosol when required. Never mix donor and acceptor membranes in the absence of cold holotransferrin even for short times, as accidental breaking of vesicles will result in content mixing and false positive signal.

The recommended order of addition of reaction components (reactions have to be set up on ice):
- water
- reagents to be tested, e.g. Rab-GDI
- cytosol (if not present in the membrane premix)
- energy (if not present in the membrane premix)
- membrane premix containing also fusion buffer and cold transferrin.

Fusion reactions are initiated by transfer of tubes from ice to 37 degrees for 25 minutes.
-Arrest reaction by adding 460 microlitres of ice cold wash buffer and vortexing.
-Pre-wash Dynabeads in wash buffer using the magnet, then resuspend 1:20 in wash buffer. Add 20 microlitres (1 bead microlitre equivalent) to each fusion reaction tube. Include a tube with 480 µl wash buffer for beads alone.
-Rotate at room temperature for 1 hour.
Wash beads using magnetic unit 2 times with wash buffer (500μl).
-Resuspend final washed beads in 100 microlitres of wash buffer containing secondary antibody conjugated to the ruthenium tag at a concentration of 200ng/100µl.
-Transfer to test tube and cover with foil. Place on shaker at room temperature for 1 hour.
-Add another 100 µl of wash buffer to each tube and then read in the Origen analyzer 1.5.