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The Microscopy-Based Assay to Study and Analyze the Recycling Endosomes using SNARE Trafficking

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TITLE:

The Microscopy-Based Assay to Study and Analyze the Recycling Endosomes using SNARE Trafficking

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SUMMARY:

Recycling endosomes are part of the endosomal tubular network. Here we present a method to quantify the dynamics of recycling endosomes using GFP-STX13 as an organelle marker.

ABSTRACT:

Recycling endosomes (REs) are tubular-vesicular organelles generated from early/sorting endosomes in all cell types. These organelles play a key role in the biogenesis of melanosomes, a lysosome-related organelle produced by melanocytes. REs deliver the melanocyte-specific cargo to premature melanosomes during their formation. Blockage in the generation of REs, observed in several mutants of Hermansky-Pudlak syndrome, results in hypopigmentation of skin, hair, and eye. Therefore, studying the dynamics (refer to number and length) of REs is useful to understand the function of these organelles in normal and disease conditions. In this study, we aim to measure the RE dynamics using a resident SNARE STX13.

INTRODUCTION:

Biosynthesis of melanin pigments occurs in melanosomes, a melanocyte-specific lysosome-related organelle (LRO) that co-exists with conventional lysosomes. The endocytic system plays a key role in the biogenesis of melanosomes, required for skin color and photoprotection against ionizing radiation[1-3]. During this process, the melanin synthesizing enzymes are sorted on early/sorting endosomes and then transported to premature melanosomes through tubular or vesicular endosomes called recycling endosomes (REs)[4-10]. The targeting and fusion of these organelles regulate the maturation of fully functional pigmented melanosomes[7,11-14]. Defects in the formation of these organelles or cargo sorting to these organelles cause oculocutaneous albinism and other clinical phenotypes, observed in Hermansky-Pudlak syndrome[15,16].

Here we describe a simple microscopy-based technique to study and analyze the REs. In this method, we have taken advantage of a transmembrane protein, Qa-SNARE Syntaxin (STX)13 that resides on recycling endosomes[17] and cycles between sorting endosomes and

melanosomes in melanocytes[12,18]. Further, deletion of N-terminal unstructured regulatory domain (namely SynN or STX13$^{\Delta 129}$) allows the SNARE to get stuck in melanosomes, which measures the forward trafficking pathway towards the melanosome[12]. We have used a known recycling endosomal marker Rab GTPase (Rab)11 in our studies[14,19]. Fluorescence imaging of the proteins GFP–STX13^{WT}, GFP–STX13$^{\Delta 129}$, mCherry-Rab11, and TYRP1 in wild type melanocytes followed by quantification of their relative localization will provide the nature and dynamics of REs in addition to their targeting to melanosomes. Thus, this is a simple technique that can be used to visualize and measure the dynamics of REs in melanocytes.

PROTOCOL:

The protocol involves the seeding of melanocytes followed by transfection of the plasmids. Further steps include fixation, immunostaining, imaging, and analysis of the cells to measure the length and number of REs. The detailed description of the protocol is given below.

1. Seeding of mouse melanocytes on pre-treated coverslips

1.1. Coat the glass coverslips in a Petri dish (i.e., 4 - 5 in a 35 mm dish) with basement membrane matrix medium (1:20 in complete RPMI medium: RPMI + 10% heat inactivated FBS + 1x Glutamine + 1x Antibiotic mix) and dry it in the tissue culture hood for 15 min. Wash the coverslips once with 1x PBS before use.

1.2. Maintain the wild type mouse melanocytes (melan-Ink4a-Arf-1 from C57BL/6J, *a/a*, *Ink4a-Arf*$^{-/-}$ mice, described in[20] and available at The Welcome Trust Functional Genomics Cell Bank) in a Petri dish (i.e., 35 or 60 mm dish) supplemented with complete RPMI medium.

1.2.1. Wash the cells twice with 1x PBS and add 0.5 or 1 mL of Trypsin-EDTA (0.25%) solution for trypsinizing cells. Incubate the cells at 37 °C for 2 - 5 min for the detachment from the Petri dish.

1.2.2. Add 1 - 2 mL of complete RPMI medium, suspend and then transfer the cells to a centrifuge tube.

1.3. Centrifuge the cell suspension at 4 °C, 376 x *g* for 5 min and then resuspend the pellet in 1x PBS.

1.4. Repeat the centrifugation step and resuspend the cells in 1 mL of complete RPMI medium.

1.5. Seed the cells on the basement membrane medium-coated coverslips at 50-60% confluency (approximately 6 x 10^5 cells on a 35 mm cell culture dish containing 4 - 5 coverslips). Always add 200 nM of PMA (add 5 µL of working stock 40 µM phorbol-12-myristate-13-acetate) to the plated cell suspension in complete RPMI medium.

1.6. After seeding, incubate the plate at 37 °C for 12- 24 h.

2. Transfection of cells with the STX13 plasmids

2.1. Use the following reagents: pEGFP-C1-STX13^{WT} and pEGFP-C1-STX13$^{\Delta 129}$ (described in[12]). mCherry-Rab11, was a kind gift from Graca Raposo, Institut Curie, Paris (described in reference[19]. Anti-TYRP1 antibody from ATCC (TA99).

2.2. After 12 - 24 h of seeding, transfect the cells with plasmids using a lipid-based transfection reagent. For a 35 mm dish, take 5 µL of the transfection reagent in 250 µL of OPTI-MEM medium in a microcentrifuge tube and take approximately 200 ng of each plasmid in 250 µL of OPTI-MEM.

2.3. Incubate the tubes containing DNA and the transfection reagent for 5 min. Mix without repeated pipetting (total volume will be about 500 µL). Incubate for 30 min at room temperature. Perform hand tapping of the tube every 10 min for 30 min at RT.

2.4. During the incubation, wash the cells twice with 1x PBS, once with OPTI-MEM and then add 1 mL of OPTI-MEM to the cells.

2.5. Post 30 min of incubation, add the transfection reagent-DNA mix to the cells in a dropwise manner by covering the dish.

2.6. Incubate the cells at 37 °C for 6 h. Aspirate the OPTI-MEM medium with transfection reagent and add complete RPMI medium supplemented with 200 nM of PMA.

2.7. Incubate the cells at 37 °C for 48 h.

3. Fixation of the cells

NOTE: The following procedure is performed outside the tissue culture hood.

3.1. After 48 h of transfection, wash the cells twice with 1x PBS and then fix the cells with 3% formaldehyde (freshly prepared in 1x PBS) for 30 min.

3.2. After fixation, wash the cells twice with 1x PBS and store the coverslips in 1x PBS until further use. Alternatively, cells can be mounted on glass slides (see below) or stored at 4 °C.

4. Immunostaining of the cells

4.1. Prepare a humid chamber: place paraffin film cut piece on moist filter paper in a Petri dish, covered with aluminum foil.

4.2. Prepare 25 µL of primary antibody solution (0.2% saponin in 1x PBS, 0.1% BSA in 1x PBS and 0.02% Sodium azide in 1x PBS). Add antibody at a dilution of 1:200. Add this solution as a drop on a paraffin film in the humid chamber.

4.3. Carefully lift the coverslip with forceps, invert it on this drop of primary antibody staining solution and then cover the lid of the humid chamber. Incubate at room temperature for 30 min.

4.4. Similarly, prepare the secondary antibody solution at a dilution of 1:500 and place it on paraffin film next to the coverslip in the humid chamber. For staining the nucleus, add DAPI (1:20,000 to 1:30,000) to the solution.

4.5. Using forceps, carefully pick up the coverslip from the primary antibody solution and dip it thrice in 1x PBS (in a glass beaker).

4.6. Tap the coverslip on tissue paper to remove the excess PBS on the coverslip. Place it on secondary antibody staining solution in the humid chamber and do not expose it to light due to the presence of fluorescently tagged antibodies in the solution.

4.7. Incubate the coverslip again for 30 min at room temperature. Please always keep a note the side of the coverslip that has the cells throughout these steps.

4.8. After the incubation, carefully pick up the coverslip from the secondary antibody solution and then dip it thrice in 1x PBS. Further, tap the coverslip on tissue paper to remove the excess PBS on the coverslip.

4.9. Place 12 µL of Fluoromount-G mounting reagent onto a glass slide and carefully place the stained coverslip (facing towards the glass) on the mounting reagent. Invert the glass slide on tissue paper and then gently press.

5. Fluorescence microscopy of the cells

5.1. Image the stained cells under Bright-field (BF) and fluorescence (IF) filters using an inverted fluorescence microscope equipped with a CCD camera using 60x (oil) apochromatic objective or any other microscope with a similar configuration.

6. Quantification of overlap between the RE localized proteins and melanosomes:

NOTE: The following steps are followed for the quantification of Mander's overlap coefficient between the proteins using Fiji software (freely downloadable from the link: https://imagej.net/software/fiji/). Use the TIFF image with multiple channels.

6.1. **Open** the raw image. Go to the **Image option,** select **Color | Split channels,** and use the two channels for analysis.

6.2. Open the **JACoP plugin** in the Plugin option.

6.3. Set the threshold for both the channels such that all the bright spots are selected and the background is eliminated.

6.4. Go to **Analysis option**, select **M1 and M2 coefficients** for getting Mander's overlap coefficient.

6.5. Press the **Analyze option** in the JACoP Plugin and see the result that displays Mander's overlap coefficient.

7. Quantification of recycling endosomes' tubular number and length:

NOTE: The following steps are followed for the quantification of the number and length of the tubules using Fiji software.

7.1. Open the raw image Go to **Image option**, select **Color | Split channels** and use the desired channel for analysis.

7.2. Go to **Image** again, select **Type | Convert** to 8-bit image.

7.3. Then go to **Plugins**, select **Analyze | Tubeness.** Set **sigma** value at 0.1075**.** Press **Ok**.

7.4. Go to **Image** again**,** select **Type | Convert** to 8 bit image.

7.5. Go to **Image | Adjust | Threshold** (Use the same threshold values for all the images. Images should have approximately equal intensity).

7.6. Go to **Process | Binary | Convert** to mask.

7.7. Go to **Process**, select **Binary** and then select **Skeletonize**.

7.8. Go to **Analyze**, select **Skeleton** and choose **Analyze Skeleton**. In **result and output |** select (a) **Calculate the largest shortest path |** (b) show **detailed information |** (c) display **labeled skeleton**. Press **Ok**.

NOTE: The result will open in the tabulated format. The column of Average branch length shows the length of all the different tubules in the cell that is selected (set scale for getting values in micrometres).

7.9. For obtaining the number of tubules in the cell, go to **Analyze,** and select **Analyze particle** option. Press **Ok**.

NOTE: In the obtained results, the count column shows the number of tubules in that particular cell.

7.10. Save the data and analyze.

REPRESENTATIVE RESULTS

Quantification of STX13$^{\Delta 129}$ mutant localization to the melanosomes

Immunofluorescence microscopy of STX13 in mouse wild type melanocytes showed GFP–STX13^{WT} localized as vesicular and tubular structures and GFP–STX13$^{\Delta 129}$ localized as ring-like structures in addition to the cell surface (**Figure 1A**). Further, intracellular ring-like GFP–STX13$^{\Delta 129}$ showed colocalization with the melanosome protein TYRP1 (**Figure 1A**) and bright-field imaged melanosomes (data not shown)[12]. As shown before, a cohort of overexpressed GFP–STX13^{WT} is observed in melanosomes[12]. To measure the relative localization of GFP–STX13^{WT} and GFP–STX13$^{\Delta 129}$ to melanosomes, we have used Fiji and analyzed with JACoP

plugin. The measured Mander's overlap coefficient (MOC) between GFP–STX13$^{\Delta 129}$ with TYRP1 is approximately 1.5 folds higher compared to GFP–STX13^{WT} with TYRP1 (**Figure 1B**). Interestingly, TYRP1 showed 2.9 folds higher MOC values with GFP–STX13$^{\Delta 129}$ compared to GFP–STX13^{WT} (**Figure 1B**). These data indicate that the localization of GFP–STX13$^{\Delta 129}$ to melanosomes is relatively higher compared to GFP–STX13^{WT} at a steady state.

Quantification of STX13^{WT} localization to the recycling endosomes

Immunofluorescence microscopy of GFP–STX13^{WT} showed colocalization with known recycling endosomal protein Rab11 (expressed as mCherry-Rab11) (**Figure 2A**,**B**). The measured MOC between GFP–STX13^{WT} with mCherry-Rab11 is approximately 1.4 folds higher compared to mCherry-Rab11 with GFP–STX13^{WT} (**Figure 2B**). To measure the number and length of GFP–STX13^{WT}-positive endosomal tubules, we have used Fiji software as described in the protocol section. mCherry-Rab11 is used as a positive control in the experiments (**Figure 2**). Melanocytes transfected with GFP–STX13^{WT} showed a higher number of tubules per cell compared to cells expressing mCherry-Rab11 (**Figure 2C** top graph, compare bar A with bar B). However, the tubule numbers are reduced upon co-expression of GFP–STX13^{WT} and mCherry-Rab11 in the cells (**Figure 2C** top graph, compare bar A with C, and bar B with D). Interestingly, the average tubule length (μm) for both GFP–STX13^{WT} and mCherry-Rab11 is comparable to each other in cells expressing individually or together (**Figure 2C** bottom graph). Together, these data suggest that GFP–STX13^{WT} localizes to REs as similar to Rab11.

FIGURE LEGENDS:

Figure 1: Localization of GFP–STX13^{WT} and GFP–STX13$^{\Delta 129}$ to melanosomes in wild type melanocytes. (**A**) Melan-Ink4a-Arf-1 melanocytes were transfected with GFP–STX13^{WT} and GFP–STX13$^{\Delta 129}$. Cells were fixed, stained with anti-TYRP1 antibody and then analyzed by fluorescence microscopy. Insets are magnified view of the white boxed areas. Arrows point to the localization of GFP–STX13^{WT} to REs and GFP–STX13$^{\Delta 129}$ to melanosomes. Scale bars, 10 μm. (**B**) Quantification of colocalization between STX13 and TYRP1. Mander's overlap coefficient between GFP–STX13^{WT} or GFP–STX13$^{\Delta 129}$ with TYRP1 and vice versa is represented (mean ± S.E.M.) separately in the plot. N=3. *p ≤0.05 and ****p≤0.0001.

Figure 2: Localization of GFP–STX13^{WT} to recycling endosomes in wild type melanocytes. (**A**) Melan-Ink4a-Arf-1 melanocytes were transfected with GFP–STX13^{WT} and mCherry-Rab11. Cells were fixed and analyzed by fluorescence microscopy. Insets are magnified view of the white boxed areas. Arrows point to the localization of GFP–STX13^{WT} to mCherry-Rab11-positive compartments. Scale bars, 10 μm. (**B**) Quantification of colocalization between STX13 and Rab11. Mander's overlap coefficient between GFP–STX13^{WT} with mCherry-Rab11 and vice versa is represented (mean ± S.E.M.) separately in the plot. N=3. ***p≤0.001. C. Quantification of number and length (in mm) of STX13- or Rab11-positive REs. The average number of tubules/cell and average tubule length of GFP–STX13^{WT} and mCherry-Rab11 are represented (mean ± S.E.M.) separately in the plot. N=3. Note that the cells are transfected with GFP–STX13^{WT} and mCherry-Rab11 in (**C**) and (**D**).

DISCUSSION:

Recycling endosomes are a cohort of endocytic organelles, and they mediate the recycling of cargo to the cell surface in all cell types[21-25]. In specialized cell types such as melanocytes, these organelles partly divert their trafficking route towards the melanosomes for their

biogenesis[3,16,26]. Further, REs are generated post cargo sorting on early/sorting endosomes and morphologically appear as tubular-vesicular structures. Their dynamics (number and length) are dependent on several cellular factors, including motor proteins[21-25,27,28]. Few studies used Rab11 as a marker to label the REs in melanocytes[14,19]. Whereas in non-melanocytes, STX13 and KIF13A have been used in addition to Rab11 to visualize and characterize REs[13,17,19]. In this study, we have used STX13 as a marker to label the REs in melanocytes followed by a comparison with Rab11-positive tubules (**Figure 2**). Interestingly, N-terminal deletion in STX13 (GFP–STX13$^{\Delta 129}$) results in mislocalization of the SNARE to melanosomes (**Figure 1**). Thus, the localization of GFP–STX13^{WT} and GFP–STX13$^{\Delta 129}$ in melanocytes visualize the REs and melanosomes, respectively. These proteins are possibly used as respective organelles markers for the steady-state localization studies.

Studies have shown that several proteins such as TYRP1, TYR (tyrosinase), VAMP7, OCA2, Rab32/38 localize to the melanosomes in addition to other intracellular organelles[3,29]. Our studies showed that the N-terminal deleted STX13 mutant (GFP–STX13$^{\Delta 129}$) localizes to melanosomes and plasma membrane. We hypothesize that GFP–STX13$^{\Delta 129}$ can possibly be used as a reporter to study the trafficking from REs to the cell surface and LRO. In contrast, GFP–STX13^{WT} localizes to REs as similar to Rab11. Our studies illustrated that GFP–STX13^{WT} could also be used for marking REs in melanocytes. We predict that GFP–STX13^{WT} may be a better RE marker than Rab11 since Rab11 overexpression alters the endosomal dynamics. Altogether, GFP–STX13^{WT} acts as a potential RE marker to study their dynamics at steady-state condition.

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DISCLOSURES:

The authors declare that they have no conflict of interest.

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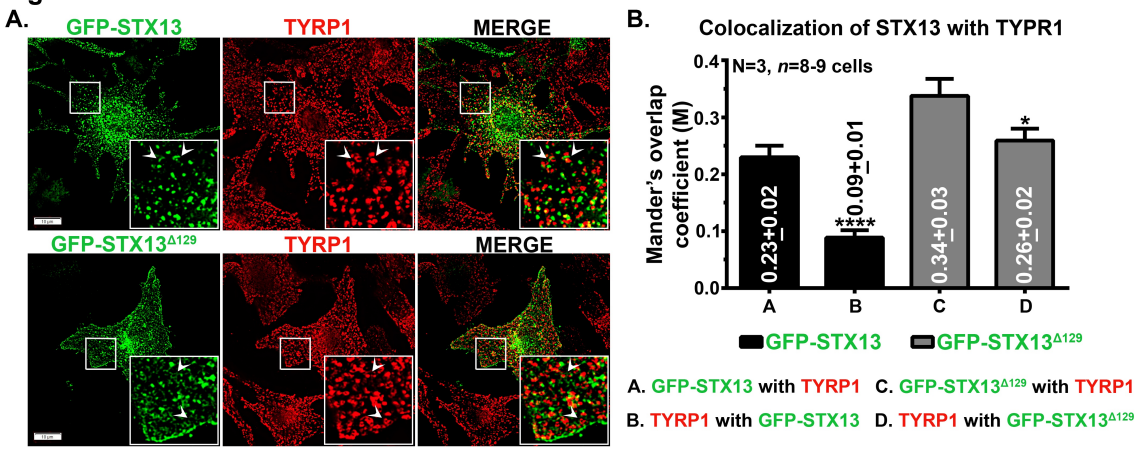
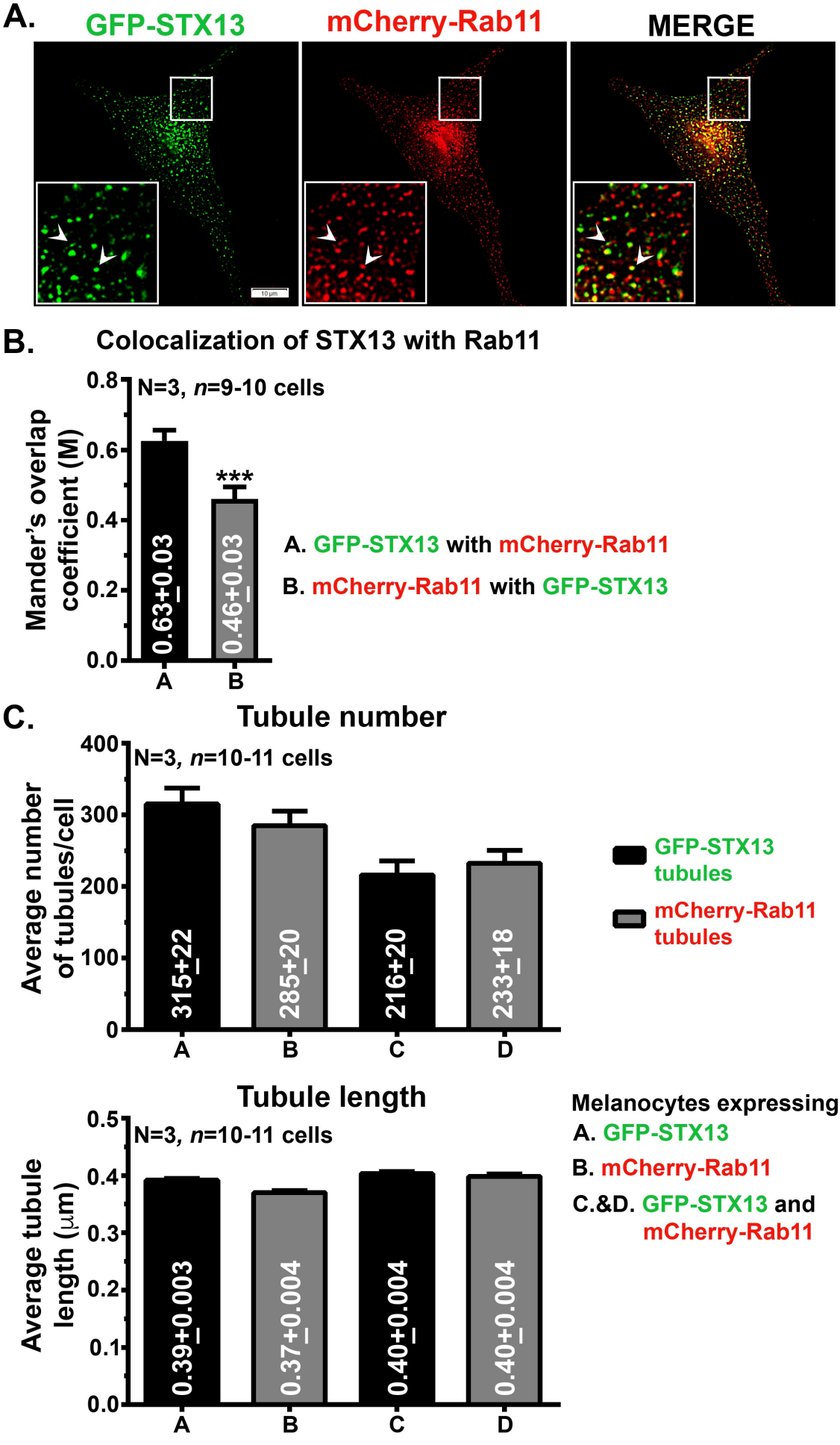
Figure 1.



A. GFP-STX13 with TYRP1 C. GFP-STX13$^{\Delta 129}$ with TYRP1

B. TYRP1 with GFP-STX13 D. TYRP1 with GFP-STX13$^{\Delta 129}$

Figure 2.


A.
GFP-STX13
mCherry-Rab11
MERGE
B.
Colocalization of STX13 with Rab11
N=3, n=9-10 cells
Mander's overlap coefficient (M)
0.8
0.6
0.4
0.2
0.0
***
0.63+_0.03
0.46+_0.03
A
B
A. GFP-STX13 with mCherry-Rab11
B. mCherry-Rab11 with GFP-STX13
C.
Tubule number
N=3, n=10-11 cells
Average number of tubules/cell
400
300
200
100
0
315+_22
285+_20
216+_20
233+_18
A
B
C
D
GFP-STX13 tubules
mCherry-Rab11 tubules
Tubule length
N=3, n=10-11 cells
Average tubule length (μm)
0.5
0.4
0.3
0.2
0.1
0.0
0.39+_0.003
0.37+_0.004
0.40+_0.004
0.40+_0.004
A
B
C
D
Melanocytes expressing:
A. GFP-STX13
B. mCherry-Rab11
C.&D. GFP-STX13 and mCherry-Rab11

Responses to the Reviewer's comments -JoVE63087R2

Responses to the Editorial message:

Your manuscript JoVE63087R3 "The Microscopy-Based Assay to Study and Analyze the Recycling Endosomes using SNARE Trafficking" has been editorially reviewed and the following comments need to be addressed before your manuscript can be formally accepted.

Response: We thank the editor for revision-2 of our manuscript. We have revised the video by including the suggested comments. We request you to expedite the review process and provide us the decision on our manuscript soon.

Responses to the Editorial comments:

Text: None – Thank you

Video:

Video editing quality, style, & pacing:

• 2:24, 2:28, Please use 15 Frame cross dissolve here (Since we have 29.9fps clips) we use half a second dissolve effect

Response: We have cross dissolve the effect at time points 2:24 and 2:28 and made the video for half a second as suggested.

• 01:48 Please check if it is possible to start this shot after the camera stops moving

Response: We have made the change in the video as suggested. In the revised video, the shot starts after the camera stops moving.