Chapter 7

Imaging "Hot-Wired" Clathrin-Mediated Endocytosis

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Abstract

Clathrin-mediated endocytosis (CME) occurs continuously at the plasma membrane of eukaryotic cells. However, when a vesicle forms and what cargo it contains are unpredictable. We recently developed a system to trigger CME on-demand. This means that we can control when endocytosis is triggered and the design means that the cargo that is internalized is predetermined. The method is called hot-wired CME because several steps and proteins are bypassed in our system. In this chapter, we describe in detail how to use the hot-wiring system to trigger endocytosis in human cell lines and how to image the vesicles that form using microscopy and finally, how to analyze those images.

Key words Clathrin, Endocytosis, Chemically induced dimerization, Live cell imaging, Immunofluorescence, Image analysis

1 Introduction

Clathrin-mediated endocytosis (CME) is a high capacity pathway for uptake of a variety of transmembrane proteins into eukaryotic cells. Constitutive CME occurs continuously at the cell surface but the initiation of each event is unpredictable. Both the timing and the contents of the vesicle are difficult to foresee [1, 2]. Previous work in vitro described how clathrin could be recruited to nucleate new pits using a clathrin-binding protein fragment attached to a planar membrane [3]. We recently expanded this work to develop a system to trigger endocytosis on-demand in living human cells [4].

During constitutive CME, a pit is initiated by a complex multistep process which in part requires the adaptor protein complex AP2 to undergo several conformational changes before it is able to engage cargo and membrane, and then recruit clathrin [5]. The system we developed removed a number of these intermediary steps such that we send a clathrin-binding protein (clathrin "hook") to a plasma membrane "anchor" to mimic the final stage

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of pit initiation. Because we have bypassed a number of steps we refer to this process of triggering endocytosis on-demand as “hot-wired” CME.

The hook and anchor are brought together by the heterodimerization of FKBP and FRB domains using rapamycin [6]. This means that both the timing of initiation and the content of the vesicle are controlled. In the basic design, the plasma membrane anchor is the α chain of CD8 to which good antibodies can be used to track internalization [7]. However, other transmembrane and even peripheral membrane proteins such as CD4, Fyn, and GAP43 can also be used. For the clathrin hook we use a fragment of the β2 subunit from the AP2 complex (hinge and appendage regions). Other hooks can be used, such as the hinge and appendage region from the β1 subunit from AP1. As a negative control, a GFP-FKBP protein is used which does not attract clathrin to the plasma membrane.

Following the dimerization of hook and anchor in response to rapamycin, there is rapid recruitment of clathrin and new clathrin-coated vesicles (CCVs) are formed. These de novo vesicles are recognizable by their intense GFP fluorescence. Their origin from the plasma membrane can also be confirmed by fluorescent antibody labeling of the anchor in live cells. We present here detailed protocols to trigger endocytosis using our hot-wiring method as well as a guide for the imaging and analysis of the vesicles generated.

2 Materials

2.1 Cell Preparation

1. Complete growth medium: DMEM supplemented with 10% FBS, 1% penicillin/streptomycin.
2. Serum-free DMEM.
3. Sterile phosphate buffered saline (PBS).
4. 0.25% trypsin–EDTA.
5. Genejuice transfection reagent (Novagen).
6. 6-well tissue culture plates.
7. Fluorodish imaging dishes (WPI).
8. Plasmid DNA—one plasma membrane “anchor,” e.g., CD8-mCherry-FRB (Addgene #100738) or CD8-dCherry-FRB (Addgene #100739) and one clathrin “hook,” e.g., FKBP-β2-GFP (Addgene #100726).

2.2 Immuno-fluorescence

1. #1.5 glass coverslips.
2. Fine forceps for coverslip handling.
3. Anti-CD8 primary antibody with or without fluorophore conjugation (Bio-Rad).

4. Green, red, far-red fluorescently labeled secondary antibodies (Life technologies).
5. Unconjugated goat anti-mouse IgG (ThermoFisher).
6. Fixation buffer: 3% paraformaldehyde, 4% sucrose in PBS.
7. Permeabilization solution: 0.1% Triton X-100 in PBS.
8. Glass microscope slides.
9. Mowiol + DAPI mounting media: 2.4 g Mowiol 4–88, 6 g glycerol, 6 ml H_2O, 12 ml 0.2 M Tris-Cl (pH 8.5), 2.5% DABCO, 10 μg/ml DAPI.

2.3 Imaging Equipment

1. Confocal microscope: Nikon Eclipse-Ti with PerkinElmer Ultraview spinning disk. Fitted with 100x oil objective, 405/488/561/640 nm lasers, 2 × Hamamatsu Orca cameras and environmental chamber set at 37 °C. Operated by Volocity software.
2. Immersion oil.
3. Leibovitz's L-15 CO_2-independent medium (no phenol red) supplemented with 10% FBS.
4. Rapamycin (Alfa Aesar) 200 μM in ethanol stock.
5. Ethanol.
6. Pipette for 100 μl.
7. Image analysis and graphing software: Fiji, Igor Pro version 7 (WaveMetrics).

3 Methods

Hot-wired endocytosis is best observed by live-cell imaging. Here, the vesicles appear as bright green spots of FKBP-β2-GFP following rapamycin addition. These vesicles can be isolated using image segmentation methods. An advantage of this technique is that it allows the dynamics of the process to be observed and there is data from the same cell before and after hot-wiring. Another way to image this process is through antibody feeding, and then visualizing the uptake in live or fixed cells. This has the advantage that the antibody-labeled anchor acts as confirmation that the bright green spots are recently derived from the plasma membrane. Ideally this is done in live cells to keep the benefits of that technique, but the number of fluorophores can be limiting. With additional immunofluorescence steps, fixed cells can be used to determine the end point of endocytosis triggered by hot-wiring. We describe each of these methods below.

3.1 Cell Preparation

We have used HeLa cells for hot-wiring endocytosis due to their ease of transfection. However, we have also used RPE1 cells and primary cultures of hippocampal neurons, we expect this protocol to be applicable to a range of cell lines. All steps are carried out in a tissue culture hood under sterile conditions.

1. *Day 1*—Seed HeLa cells in 6-well plates, 180,000 cells/well in complete growth medium, 2 ml per well.
2. *Day 2*—Transfect the cells using an appropriate method; chemical transfection with GeneJuice was used here. Mix 100 μl serum-free DMEM with 3 μl GeneJuice (per well for transfection) and incubate at room temperature for 5 min.
3. In a separate 1.5 ml tube, mix 500 ng of each plasmid DNA per well (*see* **Note 1**). Typically these are the anchor and hook constructs, CD8-mCherry-FRB and FKBP-β2-GFP (*see* **Note 2**). If a third plasmid is needed, a further 500 ng of DNA is added, with a proportionate increase in the amount of GeneJuice to 4.5 μl per well in **step 2**.
4. Combine the diluted GeneJuice and DNA, mix gently and incubate at room temperature for 15 min.
5. Replace the growth medium on the HeLa cells with fresh complete growth medium (3 ml for one well of a 6-well plate). At this stage, the cells should be between 60 and 80% confluent. Add 100 μl transfection mixture per well and mix by gentle rocking.
6. Cells are incubated with the GeneJuice-DNA mixture overnight or for a minimum of 5 h, replacing the media with 2 ml complete growth medium at the end of the incubation period.
7. *Day 3*—The cells should be split 1:3 (*see* **Note 3**) onto ethanol-sterilized coverslips in a 6 or 12 well plate, or glass-bottomed imaging dishes such as FluoroDishes.
8. *Day 4*—Cells are ready for imaging or immunofluorescence.

3.2 Live Cell Imaging of Hot-Wired Endocytosis

All imaging was performed using a 100× objective on a spinning disk confocal microscope equipped with 405 nm, 488 nm, 561 nm, and 640 nm lasers. The stage was enclosed in an environmental chamber set to 37 °C. Live cell imaging was performed using a dual camera system for simultaneous observation of GFP and mCherry excited with 488 nm and 561 nm laser lines, respectively.

1. Prepare a ready-to-use stock of rapamycin by adding 16 μl of 200 μM rapamycin (in ethanol) to 1 ml CO_2-independent medium (L-15) and keep at 37 °C. For the control, add 16 μl of 100% ethanol to 1 ml L-15.

2. Replace DMEM for 1.5 ml L-15 + 10% FBS in FluoroDishes containing HeLa transfected with CD8-mCherry-FRB and FKBP-β2-GFP (*see* **Note 4**).
3. Transfer the dish to the microscope and locate transfected cells using 488 nm (green) and 561 nm (red) lasers. Select the target cell(s), avoiding any with an atypical expression pattern (*see* **Note 5**).
4. Acquire dual color images at 5 s intervals of the chosen cell for 1 min to record a baseline for the two proteins (*see* **Note 6**).
5. Without pausing the acquisition, gently add 100 μl of the diluted rapamycin or ethanol solution using a pipette to a final concentration of 200 nM. This step triggers endocytosis.
6. Continue with image acquisition of both channels at the same frame rate until the complete response has been recorded. Successful initiation of hot-wired endocytosis results in the formation of characteristic bright GFP puncta, as shown in Fig. 1.
7. Rapamycin-induced heterodimerization is irreversible within experimental timescales so the dish is not reusable [8].

3.3 Live Cell Imaging of Antibody Labeled Hot-Wired Vesicles

1. Incubate cells expressing CD8-mCherry-FRB and FKBP-β2-GFP with 1:100 anti-CD8 conjugated to Alexa 647 (MCA1226A647, Bio-Rad) or similar far-red label in full DMEM for 30 min at 37 °C.
2. Gently wash the dish with L-15 to remove excess antibody and replace with 1.5 ml L-15 + 10% FBS.
3. Perform live cell imaging as above (*see* Subheading 3.2, **step 3** onward), with capture of additional far red (640 nm excitation) images at each time point.

3.4 Imaging Antibody Labeled Hot-Wired Vesicles in Fixed Cells

1. To HeLa cells on coverslips expressing CD8-dCherry-FRB and FKBP-β2-GFP, add 1:1000 unlabeled anti-CD8 (MCA1226, Bio-Rad) in complete growth medium at 37 °C, incubate for 30 min.
2. Trigger endocytosis by adding rapamycin to a final concentration of 200 nM and return to 37 °C. For a maximum response, incubate with rapamycin for 20 min.
3. Place cells on ice to prevent further endocytosis and wash with ice-cold complete growth medium.
4. Add Alexa 647-conjugated secondary antibody in complete growth medium for 1 h at 4 °C to label the CD8 antibody remaining at the cell surface (see **Note 7**).
5. Wash cells 3 times with ice-cold complete growth medium.
6. Optional—Add unconjugated anti-mouse IgG at 1:100 in DMEM for 30 min to saturate any remaining antibody binding

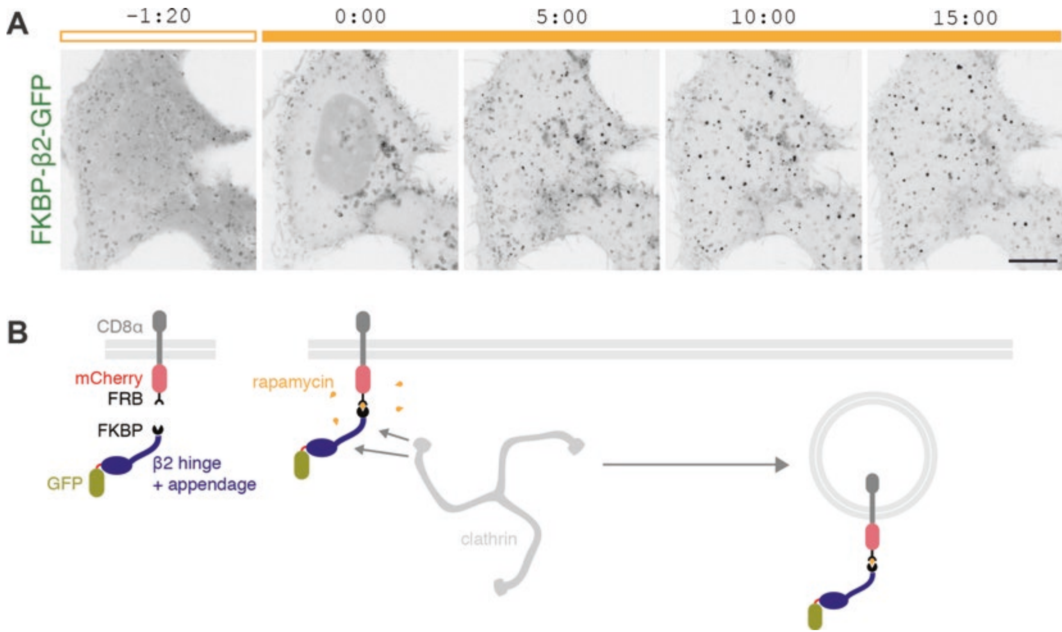

Fig. 1 Live cell imaging of hot-wired CME. **(a)** Time-lapse images showing the rerouting of FKBP-β2-GFP to CD8-mCherry-FRB at the plasma membrane following rapamycin addition at $t = 0$ min (filled bar). The number of vesicles increases over time, peaking at around 10 min. Time shown as min:sec, scale bar = 10 μm. **(b)** Schematic diagram showing the arrangement of β2 hook and CD8 anchor during the hot-wiring process. Addition of rapamycin induces dimerization of the two proteins causing the recruitment of clathrin and the formation of new clathrin-coated vesicles. Figure adapted from [4]

sites at the plasma membrane and prevent their detection in **step 9**.

7. Fix the cells using 3% PFA, 4% sucrose in PBS for 15 min.
8. Permeabilize the cells using 0.1% Triton X-100 in PBS for 10 min. Cells may now be brought to room temperature.
9. Then, add Alexa 568-conjugated secondary antibody in complete growth medium for 1 h to label any anti-CD8 that was internalized during the experiment.
10. After washing with PBS and rinsing in dH_2O, the coverslips can be mounted using Mowiol + DAPI and allowed to dry overnight before storage at 4 °C.
11. Image the slides using GFP to determine which cells were transfected and formed hot-wired vesicles. It is preferable to use 100× and take z-stacks of the entire cell volume at ~0.5 μm intervals to accurately localize the proteins.
12. The cell surface CD8 population is stained far-red, internalized CD8 originating from the plasma membrane is stained red and will colocalize with FKBP-β2-GFP. An example is shown in Fig. 2.

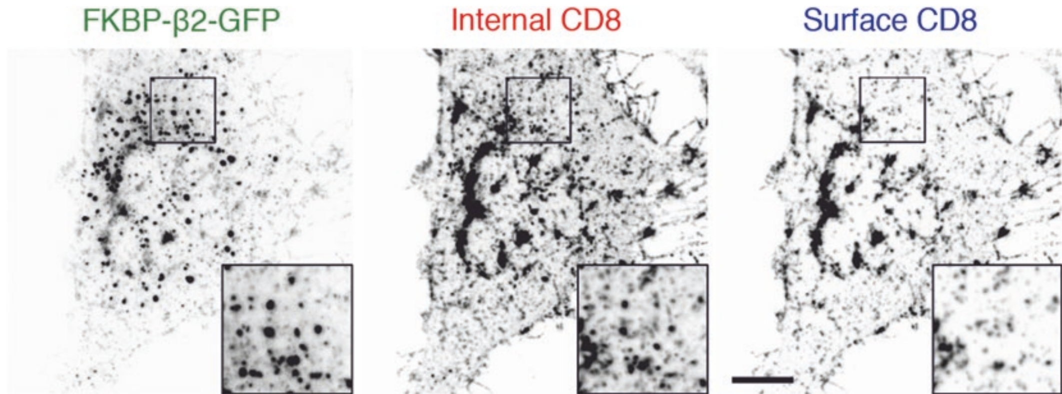

Fig. 2 Protein localization in fixed cells following antibody feeding. Confocal images of antibody uptake in response to hot-wired CME. Where there is colocalization between FKBP-β2-GFP and Internal anti-CD8, this represents an endocytic event that has originated from the plasma membrane between rapamycin addition and fixation. Where there is internal and surface anti-CD8 colocalization, this identifies the membrane CD8 population rather than internalized vesicles. Scale bar = 10 μm, inset shows 2× zoom. Figure adapted from [4]

3.5 Analysis of Live Cell Images of Hot-Wired Endocytosis

The bright green puncta of FKBP-β2-GFP which form after endocytosis is triggered can be isolated using segmentation methods. Analysis is semiautomated and performed in Fiji (Image J) and Igor Pro. Custom-written code for automated processing manually obtained Fiji outputs using Igor Pro is available here: https://github.com/quantixed/PaperCode/tree/master/Wood2017.

1. Open/Import the image stack in Fiji, the "hook" channel (FKBP-β2-GFP) is analyzed.
2. First, if significant bleaching has occurred during the movie, correct it using the simple ratio method. This improves the accuracy of the subsequent thresholding.
3. Take a small region of interest (ROI) in the cytoplasm, avoiding any obvious bright structures and the nucleus in all frames of the movie. Measure the mean pixel intensity within the ROI in each frame using the multimeasure function; the value should sharply decrease at the point of rerouting when the GFP signal moves from cytoplasm to the plasma membrane (*see* **Note 8**).
4. Draw an ROI around the perimeter of the cell and save in ROI manager.
5. Threshold the entire image to isolate only the bright green spots that form following rapamycin addition (*see* **Note 9**). This should exclude any puncta visible in the pre-rapamycin frames and also the increase in green fluorescence at the membrane following rapamycin. This will result in a binary image with the vesicles and background marked by pixels with a value of 255 and zero respectively (*see* **Note 10** and Fig. 3).

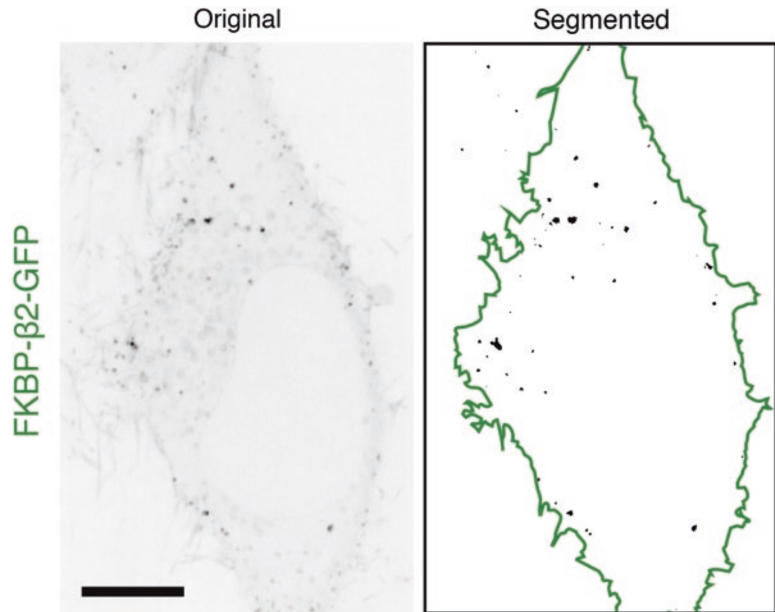

Fig. 3 Identification of hot-wired vesicles. Using thresholding it is possible to differentiate hot-wired vesicles by segmenting spots that are above a user-defined intensity. These thresholded images can then be quantified to determine the extent of vesicle formation. Figure adapted from [4]

6. Restore the whole cell ROI (from **step 4**) to this mask and use the multimeasure tool to produce data on every frame of the stack. The value used for analysis is the raw integrated density (RawIntDen) which gives the sum of the pixel values within the ROI. As all objects identified in the threshold have the same pixel value, this is equivalent to the number of pixels in these objects multiplied by 255. From the same data set, also record the area of the ROI.
7. Import into IgorPro three sets of values per cell, mean green intensity ("g wave"), RawIntDen (puncta wave or "p wave"), and area wave (constant, "a wave"). If these are stored in Excel, use a worksheet for each condition to be analyzed and use a logical naming system for each column, e.g., WT_1_g, WT_2_g, WT_1_p, WT_2_p, WT_1_a, WT_2_a. Igor will import all the data from the workbook and then present a GUI to help the user determine for every cell the time at which the green intensity has fallen by 50% (Fig. 4a). This provides the time point within the movie where the cell is halfway through the rerouting process (*see* **Note 11**). This timepoint is used for offsetting the data for averaging.
8. The code then plots the p wave vs time for each cell in a line graph. For functional clathrin hooks there is an increase following rapamycin addition, whereas for nonfunctional hooks

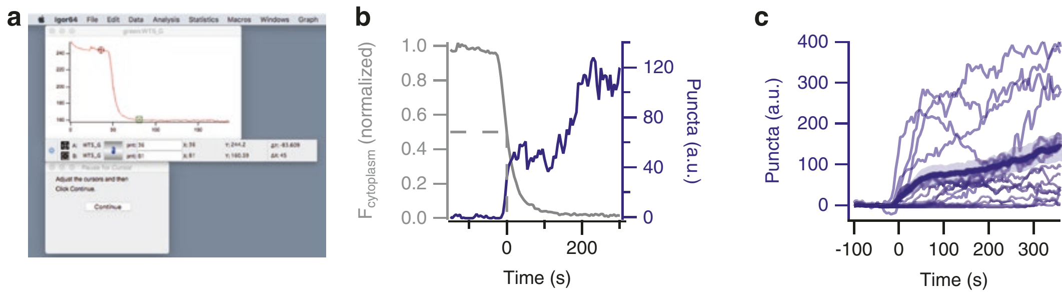

Fig. 4 Results of live-cell image analysis. (**a**) View of GUI to find the 50% rerouting point for time-alignment. (**b**) Increase in puncta over time ("p wave," right axis, dark blue), normalized to pre-rapamycin addition. The corresponding decrease in cytoplasmic GFP fluorescence ("g wave," left axis, light gray) used to time-align the puncta wave (dashed lines). Same cell as in **a**. (**c**) Multiple time-aligned, normalized puncta traces from different cells. Average ± s.e.m. is shown as a thick line with shading

and control proteins, the line stays flat. For each p wave, $t = 0$ is redefined as the time point where the cytoplasmic green fluorescence has fallen to 50%, as determined in **step 7**. This normalizes the response times from all cells to allow them to be averaged.

9. Prior to averaging, each p wave should be normalized to cell size by dividing by the area of the ROI containing the vesicles, as drawn in **step 4**.
10. The final normalization step is to adjust the y-offset of the p wave to compensate for any cells where significant background fluorescence has been picked up by the threshold. This is done by taking an average RawIntDen value from the time points before $t = 0$ and then subtracting this from all values in that p wave. Example from a single cell of normalized p and g waves are shown in Fig. 4b.
11. Having corrected for timing, area, and intensity differences, these final p wave values can be averaged to determine the mean change in RawIntDen for that group of cells (Fig. 4c).

4 Notes

1. We have a preference in our lab for Genejuice, which we find gives the highest transfection efficiency (other transfection methods can be used). When there was a large difference in the transfection efficiency of the constructs, it was beneficial to add disproportionately more of the weakly expressing protein, e.g., 250 ng + 750 ng.
2. We observed better localization of the anchor to the plasma membrane when CD8 and FRB domains were separated by mCherry. In instances where a nonfluorescent anchor was

required, a mutant of mCherry (K70N termed dark Cherry or dCherry) was used instead [9]. All alternative anchor proteins we tested worked well. For the clathrin hook we use a fragment of β2 adaptin (hinge and appendage) as this is ordinarily located at the plasma membrane as a subunit of AP2 and is known to strongly bind clathrin [10]. Again, this is exchangeable for other clathrin-binding proteins, although the ability to bind clathrin in the test-tube does not necessarily mean that the protein will work as a clathrin hook. The precise rules for how hooks engage clathrin in a functional context are currently unclear.

3. Splitting confluent transfected cells 1 well into 3 wells worked well but can be increased or decreased depending on cell confluence and transfection efficiency.
4. We prefer to image cells in phenol red-free CO_2-independent media without regulating CO_2 levels in the microscope incubation chamber. Media without phenol red reduces autofluorescence.
5. We found that allowing the dish to equilibrate for 5–10 min in the environmental chamber greatly reduced focus drift. It is best to select cells for imaging on the basis of their mCherry fluorescence. It is important to have good localization of CD8 to the plasma membrane and the minimum amount of intracellular fluorescence. Intracellular deposits of CD8-mCherry-FRB were always present although these do not prevent rerouting to the plasma membrane. Cells without a defined plasma membrane or with visible endoplasmic reticulum fluorescence should be avoided. Furthermore, rerouting efficiency of GFP signal was reduced in very dim and very bright cells, so these should also be avoided.
6. Typically, 12 images at 5 s per frame were taken prior to rapamycin addition and then a further 200 frames were taken to capture the entire response. The initial rerouting response occurs in <5 s and vesicle production peaks within the first 5 min, therefore if dynamics are required it is not recommended to acquire images slower than 10 s per frame. If vesicle tracking is required, a minimum of 2 s per frame is recommended. The illumination intensity should be kept as low as possible to reduce bleaching within the movie and prevent image saturation due to the brightness of the induced vesicles.
7. Labeling of internal anti-CD8 can also detect remaining surface binding sites, therefore wherever there is colocalization of internal and cell surface CD8 labeling; these areas are excluded from analysis. It is essential to avoid permeabilizing the cells before the surface population has been labeled.

8. The time taken for individual cells to respond to rapamycin is variable and this step is necessary if an average response is to be calculated from several cells, so that they are time-aligned to the moment of rerouting.
9. These bright spots are presumed to be hot-wired vesicles as they do not appear prior to rapamycin addition, when a non-clathrin-binding hook is used, or following clathrin siRNA treatment. They also colocalize with clathrin, anti-CD8 fed extracellularly, and by electron microscopy they represent clathrin-coated vesicles.
10. Thresholding the image can be difficult. It requires images with good dynamic range. It is easiest to isolate the bright green spots at mid-point of the movie because the final frames are often affected by bleaching. Once the minimum and maximum intensities have been determined for the chosen frame, ensure that there are not a significant number of unwanted structures in the earlier, brighter frames and that not too many GFP spots are lost in the later frames. If irregularly shaped bright objects that are too large to be hot-wired vesicles are included, Fiji's "Analyze particles…" function may be used. This will identify features of a user-defined size and circularity that closely matches the vesicles and can create a binary mask to be used in the same way as the thresholding output. Attempts to automate the thresholding task were unsuccessful due to the complexity of the images, and therefore it is essential to maintain the same inclusion criteria between samples and for the analyzer to be blind to the conditions of the images.
11. The 50% value was determined by a GUI where g waves were presented to the user and then the maxima (before rapamycin) and minima (after rapamycin) selected. These values were used to determine the time at which the midpoint was reached
12. Due to the very rapid response (within 1–2 frames), the midpoint sufficiently represents the time point of rerouting.

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