Video Article

Live Cell Imaging and 3D Analysis of Angiotensin Receptor Type 1a Trafficking in Transfected Human Embryonic Kidney Cells Using Confocal Microscopy

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Abstract

Live-cell imaging is used to simultaneously capture time-lapse images of angiotensin type 1a receptors ($AT_{1a}R$) and intracellular compartments in transfected human embryonic kidney-293 (HEK) cells following stimulation with angiotensin II (Ang II). HEK cells are transiently transfected with plasmid DNA containing $AT_{1a}R$ tagged with enhanced green fluorescent protein (EGFP). Lysosomes are identified with a red fluorescent dye. Live-cell images are captured on a laser scanning confocal microscope after Ang II stimulation and analyzed by software in three dimensions (3D, voxels) over time. Live-cell imaging enables investigations into receptor trafficking and avoids confounds associated with fixation, and in particular, the loss or artefactual displacement of EGFP-tagged membrane receptors. Thus, as individual cells are tracked through time, the subcellular localization of receptors can be imaged and measured. Images must be acquired sufficiently rapidly to capture rapid vesicle movement. Yet, at faster imaging speeds, the number of photons collected is reduced. Compromises must also be made in the selection of imaging parameters like voxel size in order to gain imaging speed. Significant applications of live-cell imaging are to study protein trafficking, migration, proliferation, cell cycle, apoptosis, autophagy and protein-protein interaction and dynamics, to name but a few.

Video Link

The video component of this article can be found at https://www.jove.com/video/55177/

Introduction

The overall goal is to obtain quantitative evidence in time and space of receptor colocalization with specific subcellular organelles after treatment with a receptor agonist. Thus, the immediate goal here is to capture time-lapse confocal images of lysosomes and a transmembrane spanning receptor in human embryonic kidney cells (HEK) following transfection and to subsequently assemble and analyze the imaging data in 3D to quantitatively measure the delivery of receptor to lysosomes. Investigating the simultaneous trafficking of a transmembrane receptor with lysosomes or other organelles during endocytosis when activated by receptor ligand can help determine how the transmembrane receptor is regulated under physiological and pathophysiological conditions.

$AT_{1a}R$ is presumed to be degraded in lysosomes following treatment with Ang II in model cells systems, primarily HEK293 [1] although the majority of receptors are primarily localized in multivesicular recycling endosomes for later recycling to the plasma membrane while the bulk of the ligand, Ang II, is degraded in the lysosome [2,3,4,5]. More recently, Li *et al.* demonstrated using Förster resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM) techniques that $AT_{1a}R$ colocalizes (on ~10 nm scale) with LAMP1, a lysosomal membrane protein suggesting that at least some of the receptor is targeted to and degraded by lysosomes [6]. These authors noted that the lysosomal inhibitor chloroquine blocked $AT_{1a}R$-LAMP1 association which is consistent with previous reports suggesting that an effect of chloroquine is to block fusion of late endosomes or autophagosomes with lysosomes. Other indirect approaches to determine $AT_{1a}R$ localization in lysosomes have utilized bafilomycin, a lysosomal inhibitor to infer $AT_{1a}R$ presence in lysosomes [3,4,5,6,7,8].

Live-cell imaging avoids potential effects of fixation on cell volume, and loss/dimming/redistribution of GFP-chimeric receptor. Previous studies have relied upon fixed cells to determine colocalization or co-occurrence of the receptor with lysosomes or using live cells labeled with receptor-binding surrogates such as β-arrestin [1,2,3,4,5,6]. Live-cell imaging of other GPCRs using spinning disk confocal or laser point scanning confocal microscopes equipped with GaASP or HyD detectors enabled investigators to observe the internalization and trafficking of receptors in individual cells imaged in z-stacks at 30 s intervals [9,10]. However even when live cell z-stacks are rapidly collected, analysis may only occur after selection of a single plane within each stack, *i.e.* in 2D [10]. While this might be sufficient for studying the internalized GPCR or tyrosine kinase receptor in question, the $AT_{1a}Rs$ accumulates in vesicles that change size and position over time and thus are inherently more difficult to adequately sample

using a representative single slice at each time point. To thoroughly determine the route of the labeled $AT_{1a}R$ through the cell and to detect each subpopulation of vesicles differing in size and position, we have devised a method for 3D imaging and 3D analysis to track these changes and to be used in conjunction with labeling of various intracellular compartments such as lysosomes.

Investigators can use this protocol for live-cell imaging to directly visualize the movement of $AT_{1a}R$ into the cell and its transfer to subcellular compartments following Ang II stimulation. Subcellular compartments are tagged with fluorescent protein chimeras or other fluorescent markers. This protocol can also be used as a first approach to localize receptors in subcellular organelles with a minimum resolution of 200 nm in order to compare mutated versus wild-type receptors or to detect changes following pharmacological treatments. The technique is accessible since it can be carried out on any confocal microscope equipped for live-cell imaging. The relative ease of this approach contrasts with increased expertise and equipment needed to carry out FRET/FLIM/BRET techniques which detect molecular interactions [6,11]. These measurements define protein-protein interactions at high resolution (~10 nm) and localization within subcellular organelles is inferred. These more advanced techniques are used to follow up and further define molecular interactions of interest, rather than the passage of receptors through subcellular compartments, and they directly demonstrate protein-protein interactions at specific times and places within the cell [11]. FLIM, a FRET technique independent of acceptor concentration, is most often performed on fixed samples since image acquisition is slower. In contrast, ratio FRET provides rapid imaging and high resolution colocalization of interacting proteins. The disadvantage of ratio FRET is that imaging utilizes widefield epi-fluorescence to gain imaging speed and to include the entire cell, resulting in reduced resolution and contrast of organelles [12]. Bioluminescence resonance energy transfer (BRET) is another advanced technique that has been applied to GPCRs to measure the acquisition of molecular proximity over time [11]. In this technique a protein fluorophore is molecularly divided and each half linked to one of two proteins in question. When the two proteins of interest bind each other, the parts of the chimeric tag re-assemble to acquire fluorescence and the increased fluorescence quantified over time.

Here, we present a straightforward technique for live-cell imaging coupled with image quantification to study trafficking of $AT_{1a}R$ in the cell upon Ang II stimulation and the potential change in delivery of internalized $AT_{1a}R$ to lysosomes following Ang II stimulation. The ultimate use for this technique is to characterize differences in membrane trafficking comparing wild type and mutated receptors. These differences will help to identify the mechanism(s) that impact physiological activities of $AT_{1a}R$ leading to regulation of blood pressure.

Protocol

Day One

1. Cell Seeding

1. Prepare complete Dulbecco's modified Eagle Medium (DMEM). Add 50 mL of fetal bovine serum, 5 mL of penicillin/streptomycin, 5 mL of L-glutamine to 450 mL of DMEM to make 500 mL of complete DMEM supplemented with fetal bovine serum (10%), penicillin/streptomycin (1%) and L-glutamine (1%).
2. Seed human embryonic kidney (HEK) cells, passage number 6-11 at 50,000/well in an 8 well chambered coverglass system in complete DMEM.
3. Maintain the chambered slide at 37 °C in a humidified incubator with 5% CO_2 for 24 h.

Day Two

2. Liposome Transfection

1. Freshly make $AT_{1a}R$-GFP plasmid and liposome solutions using sterile conditions with a biosafety level 2 cabinet.
2. Dilute 2 µL of plasmid DNA at 900 ng/µL stock solution in 178 µL of reduced serum media to achieve a concentration of plasmid DNA of approximately 10 ng/µL.
3. Dilute 4.5 µL of liposome stock solution in 175.5 µL of reduced serum media to create a 1:40 dilution of the stock.
4. Add the liposome solution to the plasmid solution and mix by using a 1 mL pipettor to pipette up and down 20 times to mix the transfection solution.
5. Incubate the mixture for 30 min at room temperature (25-27 °C).
6. While this mixture is incubating, remove the media slowly with a 1 mL pipettor and slowly add 400 µL of 1x phosphate buffered saline (PBS) that is pre-warmed to 37 °C.
7. Gently remove the PBS and replace with 200 µL/well of reduced serum media pre-warmed to 37 °C.
   NOTE: Be careful at all steps when washing the cells to avoid detachment of cells from the coverslip due to sheer stress induced by pipetting.
8. Maintain the cells at 37 °C in a humidified incubator with 5% CO_2 for 30 min.
9. Once the incubation period is over, add 80 µL of transfection mix to each well and incubate the cells for 4.0-4.5 h but no more than 5 h at 37 °C in a humidified incubator with 5% CO_2.
10. After the transfection incubation period is complete, slowly remove the media from the chambers using a 1 mL pipettor and gently replace with 300 µL/well of complete DMEM and incubate cells for 24 h at 37 °C in a humidified incubator with 5% CO_2.

Day Three

3. Serum Starvation

1. Remove the media gently using a 1 mL pipettor and slowly replace with 300 µL of serum-free DMEM without phenol red.
2. Maintain the chamber at 37 °C in a humidified incubator with 5% CO_2 for 10-12 h.

Day Four

4. Live Cell Imaging

1. **Stain intracellular compartments (lysosomes in this case).**
    1. About 30 min prior to imaging lysosomes in cells, add 22.5 µL from a 1 µM fluorescent dye stock solution (0.5 µL of 1 mM fluorescent dye diluted in 499.5 µL of serum-free DMEM without phenol red) to each well for a final dye concentration of 75 nM.
    2. Incubate for 25-30 min at 37 °C in a humidified incubator with 5% CO_2 and then gently remove the dye and slowly add 300 µL of fresh serum-free DMEM without phenol red.

2. **Prepare the microscope for imaging.**
    1. Adjust the stage down to avoid damage to the lens during startup of an automated microscope.
    2. Turn on the microscope power, scanner power, laser power and then laser emission, and finally the metal halide lamp for visual observation (see confocal microscope manufacturer instructions).
    3. Open the imaging software program and in the software turn on the argon (488 nm) laser and power up to 30-50%. Note the power depends upon age of laser. Within experiments it is crucial to maintain constant settings.
    4. Turn on red-emitting laser (*e.g.* 568 nm or 594 nm).
    5. Set imaging format to 12-bit depth.
    6. Set the pinhole for the argon laser line 488 nm and for the 568 nm or 594 nm laser to 1 Airy disk unit. If the GFP is very dim, use a setting of 2 Airy disk units, but match the channels appropriately.
    7. When using dichroic filter sets, select the appropriate emission wavelengths for GFP and the fluorescent dye. Or, set the emission wavelength (Em) at 499-580 nm for GFP and Em 599 - 680 nm for the red-emitting fluorescent dye.
    8. Check that bleed through and crossover do not occur. Turn off each laser in turn and check both emission channels for each. When the Argon laser is off, the green emission should be zero. When the green emitting laser is off, the red emission channel should be zero. NOTE: The safest method is to image each in separate tracks (sequential).
    9. Include a brightfield channel if desired. Avoid differential contrast imaging (DIC) because it can skew colocalization measurements.
    10. For live imaging, select line sequential rather than frame sequential.

3. **Select cells for imaging.**
    1. Utilize a 1.4 numerical aperture (N.A.) objective, such as a 63X/1.4 N.A. oil objective to image cells (or 40X/1.4 N.A.). Center the objective and add a drop of high viscosity, low autofluorescence immersion oil onto the objective.
    2. Transfer the chamber with the transfected cells from the incubator to the stage of the microscope. Be careful to keep the slides flat and ensure that the chamber is well seated and restrained from movement.
    3. Move the objective up until the drop of oil just touches the bottom of the slide; the right focal plane is near to this position. In automatic microscopes, save this position as well as the lowered position to use for the remainder of the imaging experiment to facilitate changing samples.
    4. Choose dim cells. Cells overexpressing AT_{1a}R-GFP will misdirect the receptor in the cell and disrupt normal function.
    5. Confirm that the outline of the cell, defined by the plasma membrane, is visible and preferably not overlapping with other cells.
    6. Focus and center the cell in the field of view.
    7. Switch to confocal mode.
    8. Select a fast imaging mode. With one hand on the focus control and the other on the stage XY control, focus and center the cell of interest visualized on the monitor.
    9. Confirm that the cell of interest is well spread and its bottom or ventral side juxtaposed closely with the coverslip by focusing up and down through the cell.

4. **Set the z-stage parameters to image a cell in 3D.**
    1. Select the On button for the Z-stack top and bottom dialog (or equivalent) and focus to the bottom of the cell then press "begin". Then, focus to the top of the cell and press "end." Recheck that the entire cell is included in the z-stack.
    2. Set the Z-step size to 1.0 µm.

5. **Set imaging settings.**
    1. Select Zoom 2, depending upon the lens, so that the final pixel size is about 0.279 µm x 0.279 µm to 0.14 µm x 0.14 µm in the XY dimension.
    2. For HEK cells in this experiment, set scan speed to approximately 1 µs per voxel and select 2 averaging or 2 accumulations by line, depending on brightness of cells.
    3. Adjust laser intensity and gain for imaging GFP and the fluorescent dye. In general, counterstains will be much brighter and therefore Photomultiplier detector (PMT) and not Gallium arsenide phosphide (GaASP) or Hybrid detectors (HyD) should be utilized for this second color channel whereas GaSP and HyD detectors are necessary for the GFP channel. If the HyD or GaASP detectors are used for the fluorescent dye, exert caution and start with the laser at an extremely low power.
    4. Set duration of scan and frequency of scanning, for example, every 30-60 s over 25 min or longer to see targeting of AT_{1a}R-GFP to lysosomes.
    5. Set the autofocus to maintain focal plane stability over time.

6. **Begin the imaging experiment.**
    1. Prepare in advance 100x Ang II stock at 5 µg/µL (4.7793 mM) and add 2.1 µL of this to 998 µL medium (10 µM).
    2. Begin ligand stimulation by the addition of 3 µL of a 100x stock solution of ligand (Ang II) to the well containing 300 µL immediately prior to imaging (final concentration 100 nM).
    3. Click start and image in 3D for the desired time and frequency.

4. Before finishing work at the microscope, collect a z-stack for later use in background subtraction during analysis. In photon counting mode, this is not necessary since the background is essentially 0. With illumination turned on and all other imaging conditions the same, but with no sample take a sample image. See Background Subtraction (below) under analysis.

Day Five

5. Image Analysis

1. **Export images to tiff format.**
   1. Right click on the image file name to see menus and select Export or click File | Export | import.
   2. Export to tiff for subsequent analyses making sure to export the RAW, 12-bit data [not the 'Red Blue Green' (RGB) conversion used for publication quality images]. Do not export to jpeg format.
   3. Note the X, Y, Z voxel dimensions and the z-step size for later use during image analysis. For example, using a 40X/1.4. N.A. objective lens these values might be 0.138 µm x 0.138 µm with 1 µm z-step size (see data in the representative results section).

2. **Create image stack for analysis.**
   1. Open Image Quantitation software (**Figure 2**)
   2. Use "Action | Create New | Images from Selected Sequence" to generate a single file image from imported single sequences (**Figure 2**, arrow #1).
   3. Enter the number of channels, the number of z-sections, and the number of time points.
   4. Depending upon the structure of the *.tiff files, select, for example, channels, z-plane, and time points as the order of the image planes. Check that the final image stack correctly represents the color channels, z-stack, and time points of the original image.
   5. Select the image name (**Figure 2**, highlighted file name at left, arrow #2). Right click on the image and/or the image field, and from the options at the bottom of the list select "Properties". In the µm pixel for (X) (Y) and (Z) size, enter the correct image properties noted above.

3. **Subtract background prior to image analysis. Background correction can be accomplished in any image analysis software. Subtract background using one of two options.**
   1. Option 1: Select "Action | Create New | Background Subtraction" (**Figure 2**, arrow #1). Use the acquired background image obtained at the end of the imaging session described above as the "Dark Reference" Image under "Tools | Subtract Background".
   2. Option 2: Inspect the darkest areas in the images where there is no sample, find the mean or maximum brightness of pixels in that region, ignoring any unusually bright pixels that could be spurious fluorophore, a randomly bright pixel, or some other phenomenon. Select "Actions | Create New | Background Subtraction". Use this pixel brightness (made negative) as the Offset.

4. **Check for color registration and correct if necessary.**
   1. Using a positive control such as imaged microbeads, or, colocalized GFP-containing vesicles and fluorescent dye (**Figure 3**) assess the need for correction by switching either the red or green color channel off and on. Visually, if registration is off by even 1 pixel, it is apparent to the eye.
   2. Generate a registration correction by selecting "Actions | Create New | Registration Correction" (**Figure 2**, arrow #1) and use the dialog box to make adjustments. Upon completion a new folder item will appear entitled "Registration Correction".
   3. Apply the registration to one or more of the images of interest by clicking the image name (**Figure 2**, highlighted file name at left, arrow #2) and then selecting "Tools | Correct Registration" (Tool button adjacent to Actions).

5. **Create measurement sequence to analyze AT_{1a}R-GFP vesicle and whole cell intensity following treatment with Ang II.**
   1. Select an image (**Figure 2**, highlighted file name at left, arrow #2) and draw a region of interest (ROI) around a single cell using the freestyle region tool (**Figure 2**, arrow #3). Use the Extended Focus mode (2D, drop down menu on upper left) to observe the cell as the cropping tool only works in 2D visualization.
   2. Right click the image and select "Crop to Selection" and a new image item is generated.
   3. Select the cropped cell by clicking on its name and then select the "Measurement" tab above (**Figure 2**, arrow #4).
   4. To define and measure internalized vesicles for GFP, drag "Find Objects" under "Finding" (**Figure 2**, arrow #5) into sequence box (**Figure 2**, arrow #6).
   5. Set "Find Objects" Channel to the GFP channel.
   6. Open settings (wheel icon in dialog box, arrow #6) and set "Threshold using:" to SD and "lower limit" to 6. Set "Minimum object size" to 0 μm^3.
      NOTE: The SD lower limit will depend on individual experiments. Visual confirmation that the correct objects are thresholded is made by examining different time points (**Figure 2**, arrow #7). The "Measurement | Feedback Options" dialog box is used to choose the way that selected thresholded objects are visualized (**Figure 2**, arrow #8).
   7. Click "Measure" in "Find Objects" dialog box (**Figure 2** arrow #6) and select the channels and type of measurements. Typically, select "All channels" and "Intensity and Volume Measurements".
   8. To define the thresholding and filters for identification of the whole cell and to measure total GFP intensity, repeat above steps (5.5.4 - 5.5.8) using the following parameters: SD 0 and "Minimum Object size" to 2 μm^3 in the second "Find Objects" dialog box to identify cells using GFP (**Figure 2**, arrow #9)
   9. Drag "Filter Population" under "Filtering" to the sequence box under population 2 (the second "Find Objects" box, **Figure 2**, arrow #9). Select "Volume (μm^3 >100).
   10. Visually ensure that only one object is identified. The entire cell should be thresholded and all other objects filtered away. The "Filter" parameter(s) may need adjustment if the cell is small, for example.
   11. Once the measurement has been standardized, save the protocol by right clicking the image and select "Save Protocol" in order to reuse ("Restore Protocol"). The protocol can also be exported to provide a record in the same subdirectory as the image.

12. Proceed to "Make Measurement" by selecting "Measurements | Make Measurement Item" (**Figure 2**, arrow #8) or right clicking the image and select "Make Measurement" and "All Timepoints".
13. Enter the correct measurement name (copy paste from the image is convenient) and analysis code or date (a helpful addition for record keeping) and select "All Timepoints". A new worksheet item will be created beneath the image. Keep file names concise.

6. **Analyze and export the data.**
    1. Click on the worksheet file name and select the "Analyze" tab. Determine if saturated voxels are present by first selecting "Restrict Analysis to:" select Population 2 (the cell).
    2. Right click the field under the "Analyze tab" and in the dialog box under "Analyze these data:" select "Max ([GFP color channel])"; under "Summarized by:" select "Mean ([GFP color channel]"; and under "Organize the data by:" and "Row" select "Timepoint" and click "OK".
    NOTE: If data contains saturated pixels (*e.g.* 65,536 for 16-bit or 4,096 for 12-bit), the analysis will not be accurate (it will underestimate vesicle content). Disregard that cell for analysis. If not saturated, proceed to the next step.
    3. Next, under the “Raw” tab and then under the “Analysis” tab and right-clicking the data find "Restrict Analysis to:" then select Population 1 for vesicles or Population 2 for the whole cell.
    4. Under "Analyze these data:" select "Sum" (GFP color channel); under "Summarized by:" select "Sum"; and, under "Organize the data by:" and "Row" select "Timepoint" or "Relative Time" and then click "OK". Analysis selections may be saved and reused.
    5. Select and copy/paste directly from image quantitation software to a blank spreadsheet or export the spreadsheet data by right clicking the data and selecting save as .csv file. The whole cell intensity measurement will be included with measurements of each vesicle in the population so be sure to identify and separate it from the vesicles.
    NOTE: After plotting the data, the increase in total GFP fluorescence contained within vesicles rapidly rises. Photobleaching, if it occurs, is detected as loss of total cell GFP intensity over time.

7. **Create measurement sequence to analyze GFP present in lysosomes.**
    1. In the Find Objects (Population 1), set "Find Objects" Channel to the red channel and in wheel icon within that dialog box indicated by arrow #6 (**Figure 2**) set "Threshold using:" to SD and "lower limit" to 6. Set "Minimum object size" to 0.017 μm^3.
    NOTE: The SD lower limit will depend on individual experiments. Visual confirmation that the correct objects are being thresholded should be made by examining different time points. The "Measurement | Feedback Options" dialog box is used to choose the way that selected thresholded objects are visualized (**Figure 2**, arrow #8).
    2. Click "Measure" in "Find "Objects" dialog (**Figure 2** arrow # 6) and select the channels and types of measurements. Typically, "All channels" and "Intensity and Volume Measurements" are selected.
    3. For Population 2, use settings identical to that used to identify the whole transfected cell expressing AT_{1a}R-GFP (5.5.9).
    4. If multiple cells (including untransfected cells with fluorescent dye-positive vesicles) are present, cropping the cell at the first stage of analysis is critical. Alternatively, the use of "Compartmentalize" should be used to limit identification of lysosomes within the transfected cell expressing AT_{1a}R-GFP. Lysosomes must only be identified within the GFP cell and not from nearby, untransfected cells.
    5. Analyze and export data as above.
    NOTE: An example of thresholded cells and vesicles is shown in **Figure 4**.

Representative Results

In this representative set of experiments, HEK cells were transfected with angiotensin type 1a receptor (AT_{1a}R-GFP) construct (E1,2,3-AT_{1a}R) cloned into the pEGFP-N2 plasmid[13]. Time-lapse images of HEK were acquired and played as a movie (See **Live Cell Imaging 1** and **Live Cell Imaging 2 movies**). The movies and stills show that after ligand stimulation, AT_{1a}R-EGFPs are localized predominantly in the plasma membrane, but with the addition of Ang II these receptors become internalized within 2.5 min to form bright vesicles and at later times cluster in larger vesicles in a perinuclear zone (**Figures 4B, 5,** and **6A; Live Cell Imaging 2**).

Quantification of total cell GFP intensity and total vesicle GFP intensity for the cell in **Figure 5** provide an illustration of the complications inherent when Ang II induces ruffling and cell shape changes immediately following stimulation (**Figure 5A**, arrow at 0 min post-Ang II, upper row, orange indicates thresholded objects). At later times, the localization of GFP-containing vesicles in a perinuclear cluster also interferes (**Figure 5A**, red arrow at 29.5 min). Although all objects should be included in the total cell GFP intensity measurement, the ruffles should be excluded from vesicle measurements as they represent plasma membrane AT_{1a}R-GFP. In contrast, the clustered perinuclear vesicles should not be excluded from the vesicle measurement (the latter are seen in an area of the cell enclosed in red circles in **Figure 5A**, arrow at 29.5 min post-Ang II, lower row). Attempts to accomplish this by size filtering (more than or less than 7 μm^3) was not useful as it could not discriminate ruffles and clustered vesicles (**Figures 5A** and **5B**, compare Filter>7, Filter<7, and no filter). A possible alternative would be to draw a Regions of Interest (ROI) encompassing the cytoplasmic portion of the cell but excluding the ruffling edge and perimeter of the cell. Ultimately, this cell would not be included in quantitative results because there were saturated pixels of GFP present.

A second representative example is shown in **Figure 6A** illustrating a time course in which the autofocus was not used. The internalized GFP is shown as percent total cellular GFP (**Figure 6B**). The time points prior to the red arrow at 3 min are misleading as only part of the cell was in the imaging volume but 3 min after Ang II addition, the focal plane restabilized. The total GFP within the cell demonstrates that this cell did not photobleach appreciably over time (**Figure 6C**). Between 3 and approximately 12 min, percent GFP intensity in vesicles increases then levels off (**Figure 6B**). Next, lysosomes identified by fluorescent dye were used to identify ROIs within a transfected cell which were then quantified for the sum of GFP intensity (**Figure 6D**). After three minutes, the intensity of AT_{1a}R-GFP in red-identified lysosomes does not vary over time (**Figure 3D**, n = 5, mean ± standard error of the mean). Thus there is no detectable increase in delivery of AT_{1a}R-GFPs to the lysosomes for up to 24 minutes after Ang II administration.

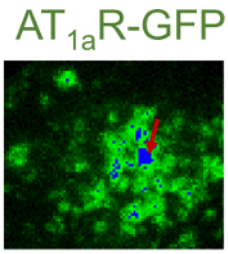


Figure 1: Saturated Pixels Identified in a Lookup Table (LUT). The red arrow indicates saturated pixels shown in blue. Please click here to view a larger version of this figure.

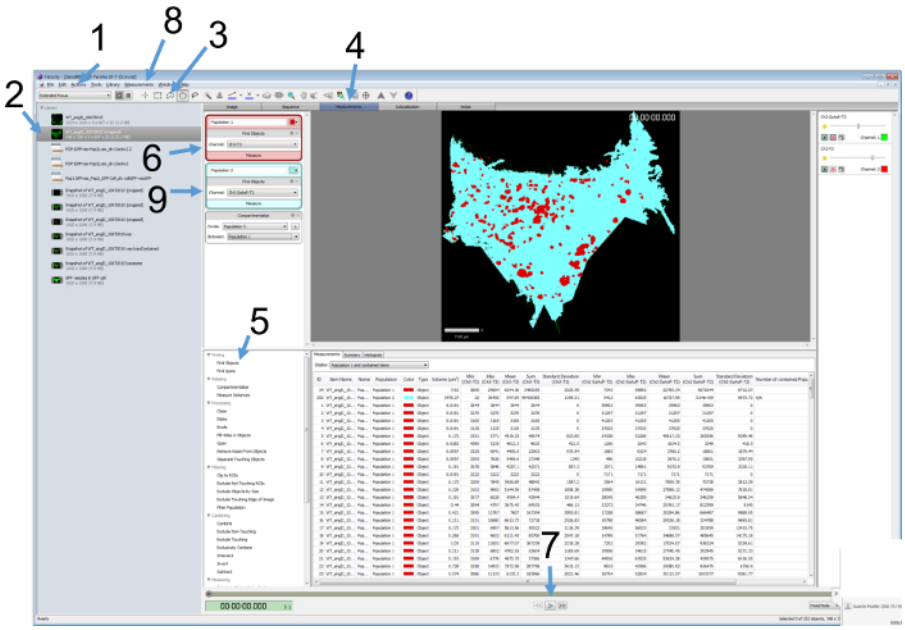


Figure 2: Overview of Image Quantification Software. Key items are indicated by numbers and discussed within the methods text. 1) Action item, 2) Selected image, 3) free-hand ROI tool, 4) Measurements tab, 5) Find Objects, 6) Population 1 Find Objects dialog box, 7) Arrow activating time lapse movie, 8) Measurements item, 9) A second Find Objects dialog box containing a Filter Population command. Please click here to view a larger version of this figure.

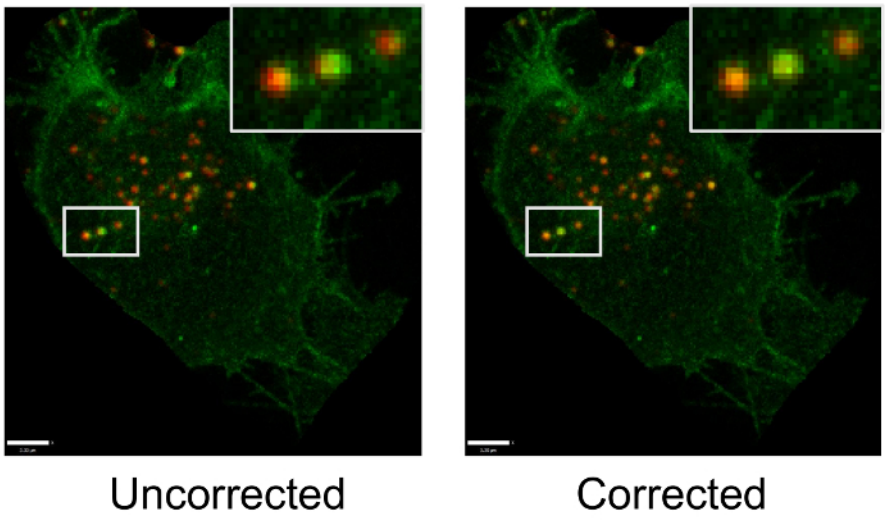


Figure 3: Color Registration Correction. Images with uncorrected and corrected pixel registration are shown together with insets of several colocalizing vesicles containing AT_{1a}R-GFP. The red pixels are shifted 1 pixel to the left in the XY plane of the uncorrected image. Insets show boxed areas at higher magnification. Scale bar = 3.3 µm, voxel dimensions = 0.138 x 0.138 x 1 μm^3. Please click here to view a larger version of this figure.

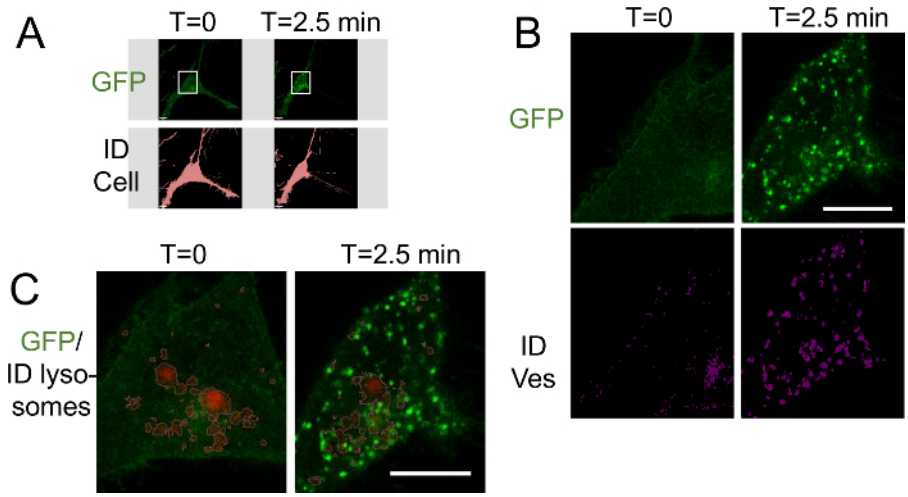


Figure 4: Illustration of Thresholding to Identify GFP- and Fluorescent Dye-containing Vesicles and GFP-whole Cells. (**A**) Identification of whole cells using AT_{1a}R-GFP (GFP, top row) and thresholded GFP for the whole cell (ID Cell, bottom row) are shown at 0 and 2.5 min after Ang II addition. White boxes indicate inset areas shown in (**B**) and (**C**). (B) AT_{1a}R-GFPs are compared at 0 and 2. 5 min after Ang II addition in single color images (top row). Vesicles identified by thresholding are shown in the bottom row (ID Ves, purple). (**C**) Two-color images (AT_{1a}R-GFP /fluorescent dye) are shown at 0 and 2.5 min post Ang II addition. Lysosomes identified by thresholding are shown by red outlines. Scale bar = 7.0 µm, voxel dimensions = 0.114 x 0.114 x 1.4 μm^3. Please click here to view a larger version of this figure.

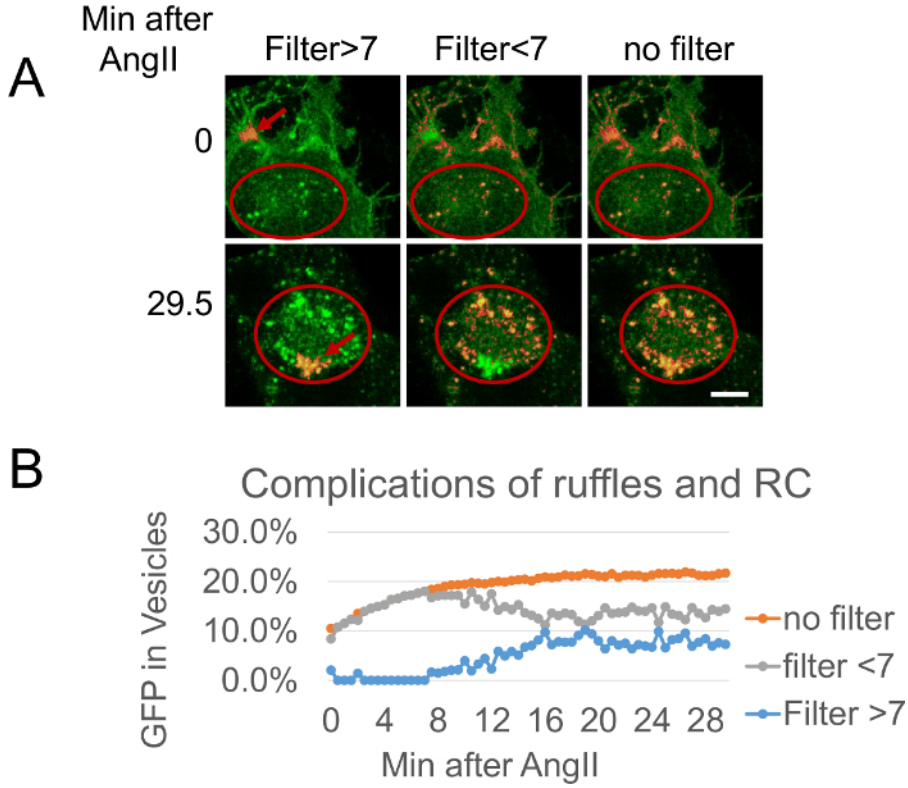


Figure 5: Illustration of the Effects of Ruffles and Clustered Vesicles on Measurement of Internalized GFP Receptors. (**A**) AT_{1a}R-GFP in Ang II treated cells at 0 (top row) and 2.5 min (bottom row) are shown in three filtering experiments where in the first column a filter was applied to select only objects greater than 7 μm^3, in the second column to select objects less 7 μm^3, and in the third column no filter was used. Thresholding level was constant in all three. Arrows indicate large thresholded objects, circles indicate an area of cytoplasm containing perinuclear vesicles at later time points. Thresholded areas are orange. (**B**) Quantification of GFP total intensity in vesicles is shown for thresholded areas with or without size filtering (by column in A) at two time points (by row in A). Voxel dimensions were 0.138 x 0.138 x 1 μm^3. Scale bar = 5.6 µm. Please click here to view a larger version of this figure.

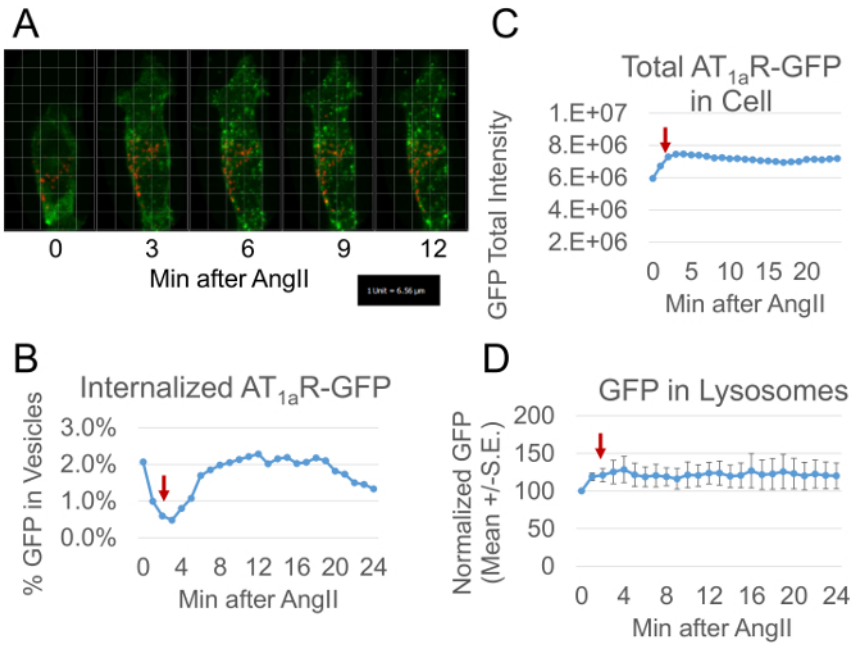
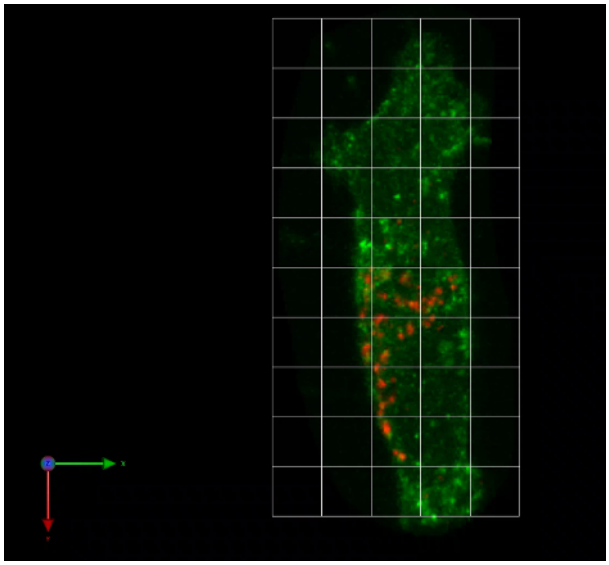
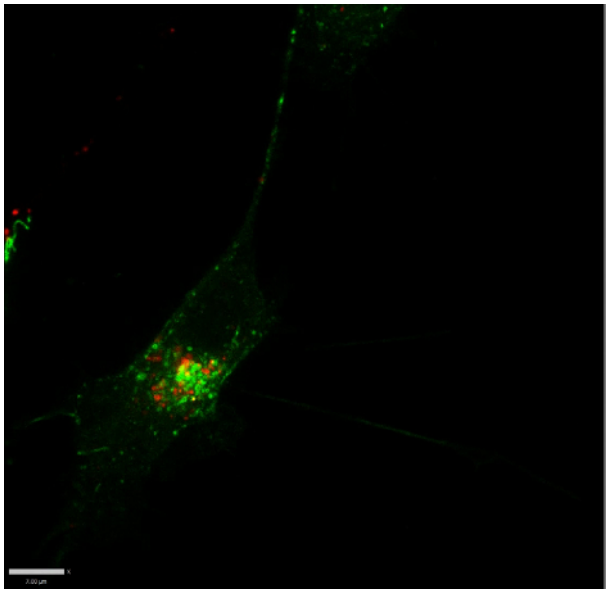


Figure 6: Tracking AT1$_a$R- GFP Internalization and Potential Localization in Lysosomes. (**A**) Five selected time points of AT$_{1a}$R-GFP with LysoTracker Red are shown from the accompanying movie (Live cell imaging 1 movie) at 0, 3, 6, 9, and 12 min post Ang II. The grid represents a unit scale of 6.56 µm^3, voxel dimensions were 0.297 x 0.297 x 1.2 µm^3, and the z-step = 1.2 µm. (**B**) A plot of the percent total GFP in vesicles over time illustrates the rapid internalization of AT$_{1a}$R-GFP. (**C**) A plot of the total GFP in the cell over time illustrates that relatively little photobleaching occurred during 24 min of imaging. (**D**) Lysosomes were identified by identifying LysoTracker Red-positive vesicles. The total GFP intensity within fluorescent dye-positive vesicle ROIs was measured at each time point (Normalized to the first time point with no compensation for photobleaching which was not independently measured). (**C**-**D**) Prior to the arrow, the measurements are not valid since the focus shifted. N = 5, Mean ± standard error of the mean. Please click here to view a larger version of this figure.



Movie 1: Live Cell Imaging 1: (Right click to download) HEK cell transfected with AT$_{1a}$R-GFP was imaged every 1 min for 24 min (25 frames) after the addition of 100 nM Ang II. Movie was acquired using a laser scanning confocal microscope with a Plan-Apochromat 63X/1.4 N.A oil objective, voxel dimensions of 0.279 x 0.279 x 1.2 µm^3; and 5 slices for a total of 4.794 µm thickness imaged at 3.20 µs/pixel. The movie rate is 2 frames/s. Unit = 6.56 µm.



Movie 2: Live Cell Imaging 2: (Right click to download) HEK cell transfected with $AT_{1a}R$-GFP was imaged every 30 s for 25 min (51 frames) after the addition of 100 nM Ang II. Movie was acquired using a laser scanning confocal microscope with a Plan-Apochromat 40X/1.4 N.A oil objective. Voxel dimensions were 0.11 x 0.11 x 1.4 μm^3. 7 slices for a total of 8.4 µm thickness were imaged at 3.72 s/frame. The movie rate is 3 frames/s. Scale bar = 7 µm.

Discussion

Preliminary experiments presented here illustrate that over a rather short time course of 24 min, no appreciable change in delivery of $AT_{1a}R$-GFP to the lysosomes occurred. In general, delivery of internalized receptors to lysosomes could occur as early as 15-20 min. This experiment is consistent with the hypothesis that the bulk of the internalized $AT_{1a}R$ is targeted to large, perinuclear endosomes. Evidence that at least some $AT_{1a}R$ arrives in lysosomes was demonstrated by Li *et al.*, who compared cells at 5 min and 30 min after Ang II treatment and found significant colocalization of LAMP1 with $AT_{1a}R$ at 30 but not 5 min [6].

This paper provides a detailed description of cell preparation, imaging, and analysis steps and discusses the many inherent pitfalls in this advanced imaging technique. This method depends upon critical steps discussed below.

Cell Culture and Transfection

Since the good health of cells is critical for any live cell imaging experiment, it is important to avoid using cells beyond passage 11 because of changes in growth rates and transfection efficiency at higher passages. To maintain healthy cells throughout the experiment, the incubation period of transfection mix with cells should not exceed 5 h since liposome solution is toxic to cells evidenced by increased membrane blebbing. Temperature, humidity and CO_2 control is significant before as well as during imaging. Drifts in temperature during imaging can cause relatively major z-section drifts and loss of focal plane stability. Hence extra caution is recommended during addition of Ang II to the cells during imaging to ensure that the focal plane is accurately maintained.

Imaging

An advantage of live-cell imaging over immunostaining using fixed cells is that the *in vivo* conditions can be closely controlled and artifacts associated with fixed cells can be avoided. However, live-cell imaging can be challenging to optimize for cellular processes that are extremely rapid, like receptor internalization. Instrument speed and the need to avoid photobleaching limit the choice of events that can be studied. Each imaging experiment might demand certain choices to be made in some parameters and compromises in others; examples are choices in pixel size, line accumulation, zoom factor, and scanning speed to get good quality data for analysis and avoid oversampling at the same time. Critical decisions in these factors depend upon balancing the need for obtaining adequate pixel size to distinguish individual vesicles and the need for obtaining sufficient temporal resolution of the events that occur. Thus some tradeoffs must be made in order to acquire information quickly enough as the cell begins to internalize GFP-labeled receptor. In our case, although modern confocal software can suggest the optimal pixel size, our experiments used a value less than the optimal pixel size according to the Nyquist equation [14]. Optimal choices of settings for other experiments will also depend upon how bright the cells are since larger pixel size will enable faster accumulation of photons allowing a trade off with longer voxel dwell time to collect more photons.

For the analysis to be successful, the live-cell imaging also critically requires accurate focal stabilization during time-lapse imaging. Focal shifts as well as failure to image the entire cell can have profound impact upon quantification of receptor internalization as well as total cellular GFP intensity. The solution to this problem of focal shifts during earliest time points of imaging following the addition of Ang II has been provided by confocal manufacturers in the form of autofocus solutions whereby coverslip interferometry with an IR laser coupled with feedback to automated focus control is used to maintain relative distance from the objective to the coverslip surface identified by the IR reflection (*e.g.* Definite Focus or Adaptive Focus Control). The remaining problem of the microscopist is then to identify the correct z-range so that despite shape changes, the entire cell remains in the imaging volume. Temperature is another critical factor to maintain the focus/Z position during initial time points

after addition of Ang II. The temperature of the objective as well as the live cell imaging chamber should be maintained at 37 °C throughout the experiment.

Another critical component of imaging, is acquiring images quantitatively. If a HyD detector is used in the Photon Counting Mode, the imaging parameters must be set so that photon pileup does not occur. For PMTs and other detectors, the images must not contain saturated pixels (voxels). In both cases, a specialized look-up table (LUT) can be used to visualize the presence of saturated pixels and of course if they are present, the microscopist should reduce laser intensity and/or gain.

Analysis

Although the approach described here is applied to the $AT_{1a}R$ receptor and its colocalization with lysosomes, it is applicable to experiments in which the relative quantity of the receptor in subcellular compartments labeled with various fluorescent tags is at question. If the goal is to measure whether or not the receptor moves to the organelle, then using a Pearson's colocalization coefficient with a marker for the organelle is problematic. Every pixel where the organelle is identified will have a high amount of the marker so that one of the coefficients will always be near 100%. Thus, colocalization measurements could be misleading. On the other hand, measuring the percent of receptor on the organelle versus total amount of receptor is much more informative and clearer to interpret. A case in which protein colocalization might best be described using Pearson's correlation coefficient would be $AT_{1a}R$ receptor and LAMP1 whose interaction has been documented, albeit by a more sophisticated colocalization approach, namely FLIM/FRET [6].

The use of 3D imaging is more accurate than using representative slices of cells with analysis in 2D because of cellular movements that are inevitable during the time course (**Live Imaging 2 movie**). It is clear from the live-cell images shown in **Figures 3, 4B,** and **5A** that the receptor is primarily localized at the plasma membrane, however, within a few minutes of exposure to Ang II it localizes to bright spots on the membrane and small vesicles adjacent to the membrane, probably coated pits and early endosomes (**Figures 4B**, **4C**). With continued progression of time, the vesicles move through the cell accumulating in larger vesicles adjacent to the nucleus that have been described as multivesicular endosomes (MVEs, **Figures 1, 5A**) [3,15]. A single slice through the cell over time would distort the internalization process and misrepresent the true events particularly if the slice did not include the cell surface at early times, and the MVEs at later times.

In this study, two approaches for image analysis can minimize effects of photobleaching of GFP as well as fluorescent dye on the quantitative outcome. As cells photobleach (albeit to a limited extent), the relative brightness of internalized vesicles relative to the entire cell intensity is maintained, assuming photobleaching occurs uniformly. Fluorescent dye-positive vesicles are also easily identified by selecting the standard deviation of the mean fluorescent intensity option for thresholding bright internalized vesicles. This distinguishes them from background even as the entire level of fluorescence declines. Thus, our measurements were not sensitive to the effects of modest photobleaching. However, strong photobleaching such as 50% loss of cell intensity over time might have a larger impact upon quantitative results. Alternatively, the effects of photobleaching could be measured independently in control experiments absent Ang II in the case of $AT_{1a}R$-GFP or in adjacent untransfected cells in the case of fluorescent dyes. The effects of photobleaching could then be factored in during analysis.

Additional Concerns

Recently, cell biologists have conducted critical assessment and modification of fluorescent proteins for monitoring membrane trafficking in cells to avoid artifacts generated by the fluorescent protein itself, independent of the protein being tagged. Various fluorescent proteins are vulnerable to interactions with the local microenvironment leading to protein precipitation or crosslinking based on their pKa's and presence of the amino acid cysteine, respectively [16]. Another outcome of these interactions may include loss of fluorescence [16]. All of these artifacts affect quantitative measurements of trafficking. Constantini *et al.* have generated monomeric fluorescent proteins for imaging that lack cysteine to eliminate potential artifacts [16]. Thus, depending upon the goal of the imaging experiments, switching from EGFP (non-monomeric) to a monomeric, cysteine-free form would be advantageous for studies of acidic and/or reducing vesicular microenvironments. Another example would be their improved utility for FRAP or FRET experiments where multimerization and crosslinking would impact protein diffusion and protein-protein interactions.

An additional caveat is that it is important to test whether or not EGFP labeling modifies the normal behavior of the receptor. One way to test this possibility is by determining if there are any differences in receptor affinity (Kd) and maximum receptor number (Bmax) by conducting radioligand binding assays on cells transfected with the wild type or the EGFP-tagged receptor.

Another potential artifact to consider when imaging lysosomes using LysoTracker Red together with GFP fusion proteins is that LysoTracker Red has been shown to be capable of photoconversion to green [17]. The authors recommend that use of epifluorescence illumination be minimized, discuss occurrences of this artifact, and refer to successful instances of colocalization using this reagent. Additionally, other markers of the lysosomes can also be employed to verify colocalization results, for example, tagged LAMP1 [6].

In future, the sensitivity and specificity of this assay can be examined for receptor targeting to lysosomes as well as other subcellular compartments by using inhibitors of vesicle transport and/or fusion, by inhibiting lysosome acidification using bafilomycin to block degradation but not accumulation [7,18], by using chloroquine to block fusion with lysosomes consequently blocking co-localization of receptor with lysosomes [2,5,6,7,18], by using positive controls such as the targeting of labelled Ang II to lysosomes [2], and by comparison to other well-described receptor and ligand systems such as transferrin or epidermal growth factor and their cognate receptors [3]. An interesting positive control, albeit artefactual, in our experiments was a result observed in adjacent, untransfected, $AT_{1a}R$-GFP negative cells. These cells apparently took up GFP released from transfected cells and targeted it to lysosomes where colocalization with fluorescent dye was quite prominent (not shown). This colocalization, however, was not sensitive to Ang II as expected.

In conclusion, the methods for transfection, imaging, and image analysis presented here provide a means by which receptor internalization to various subcellular compartments can be quantitatively traced within living cells over time. Relative comparisons between different receptors or selectively mutated receptors are possible with regard to kinetics and subcellular localization to specific compartments. Possible difficulties and artifacts associated with this technology are discussed. Nonetheless, fast live-imaging in 3D coupled with image analysis can be used

to measure receptor uptake and rapid transitions through the cytoplasm to molecularly definable compartments. This sets the stage for measurement of differences resulting from mutation of receptor or application of agonists, antagonists, and inhibitors.

Disclosures

The authors declare that they have no competing financial interests.

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