Supplementary Methods - detailed protocol

GFP recognition after bleaching (GRAB)

General remarks
An experiment can usually be performed in three days. Day 1: Seeding of cells into glass bottom culture dishes (e.g. MatTek). Day 2: Fluorescence microscopy, fixation, pre-incubation steps, GFP bleaching, post-fixation, dehydration and embedding in resin. Day 3: Removal of bottom glass, ultrathin sectioning, electron microscopy and data evaluation. It is recommended, however, to insert one day in between each ‘experimental day’ for the proper attachment of cells (after day 1) and the correlative step (after day 2). Experimental day 2 could be divided into two ‘experimental days’ after the osmium post-fixation step.

Required skills are basic experience in fluorescence microscopy, cytochemical sample preparation and electron microscopy. To avoid false positive or false negative GFP ultrastructure localization, a detailed fluorescence microscopic evaluation of the GFP localization should preferably be undertaken before examining the sample at the ultrastructural level.

Control experiments without illumination and mock experiments without GFP expression should always be included, as well as positive controls. This will minimize the risk of misinterpretations. The protocol as described below is developed using stably transfected human cell lines (HeLa) expressing EGFP or ECFP fusion proteins. Adaptation to other fluorescent proteins, cell types or organisms may require optimization.

Caution: Some chemicals used in this protocol are highly toxic and dangerous. Necessary precautions like safe storage, use of fume hood and gloves should therefore be taken, including disposal of what is effectively toxic waste.

Day 1: Cell culture
Cells are seeded in uncoated glass bottom (No. 1.5) Petri dishes (MatTek) 24 h to 48 h before use. For correlative microscopy, the ‘CELLocate’ or ‘grid’ glass bottom dishes are recommended (MatTek), but generally, the bleached area can be detected after osmication and resin embedding using a binocular microscope. Alternatively, ‘landmarks’ on the glass bottom can be scratched
using a diamond pen, followed by UV-light sterilization before seeding the cells. HeLa cells (No. CCL 185; American Type Culture Collection, Rockville, MD) will grow in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 2 mM L-glutamine (GIBCO BRL, Life technologies) at 37°C and 5% CO₂. For correlative microscopy, the cells should be grown in loose groups and not as a confluent monolayer, so that a given cell can be identified later by its individual shape. Alternative correlative identification techniques can be used as well, in conjunction with a confluent monolayer (for example, with polarized cells) or tissue.

**Day 2: Fixation, bleaching, EM preparation**
All incubation steps including fixation, bleaching, dehydration and resin polymerization are carried out in the glass bottom tissue culture dishes.
1. Take cells from incubator and wash immediately with pre-warmed (37°C) calcium- and magnesium-free phosphate-buffered saline (PBS) pH 7.4 for 30 s.
2. Fix cells with pre-warmed (37°C) fixative containing 2% glutaraldehyde (25% stock solution, Merck) and 2% sucrose (USB) in PBS for 30 min.
3. Wash samples 3 x 5 min with PBS.
4. Block endogenous enzyme activity with 50 – 100 mM potassium cyanide and 100 mM glycine in PBS for 1.5 h.
5. Block endogenous autofluorescence by 100 mM ammonium chloride in PBS for 40 min.
6. Block endogenous autofluorescence by 10 mg/ml sodium borohydrate freshly dissolved in PBS for 40 min.
Potassium cyanide eliminates background DAB oxidation that occurs as a consequence of mitochondrial respiration. Glycine, ammonium chloride and sodium borohydrate reduce glutaraldehyde autofluorescence and consequently, background DAB precipitation.
7. Wash samples 3 x 5 min in Tris-buffered saline (TBS) at ph 7.4.
8. Photo-oxidation: samples are incubated in a solution of 1- 2 mg/ml 3,3'-diaminobenzidine hexahydrate (DAB)(Polysciences) in TBS. The DAB solution should be freshly prepared, kept on ice in the dark, and only be used on the same day. Immediately before applying to the samples, the DAB solution is oxygen enriched by bubbling through pure oxygen gas. If a 2 mg/ml
DAB concentration reversebly quenches GFP fluorescence, the concentration should be lowered to 1.5 or 1 mg/ml DAB. Only a well visible GFP fluorescence at this stage will result in effective photo-oxidation.

To bleach, samples are illuminated with the appropriate filter settings for EGFP (exitation filter BP 470/40) or ECFP (exitation filter BP 436/20) using a 100 W mercury lamp (AttoArc by Carl Zeiss) on an inverted microscope fitted with a high numerical aperture objective (63x planapochromat 1.4 NA). The climate chamber of the microscope should be set to the lowest temperature (below 10°C) for effective DAB precipitation. Alternatively, a specimen cooling chamber can be used (Temperable Insert P, Leica). The lower temperature, the higher is the oxygen saturation and therefore photo-oxidation efficiency. The development of DAB reaction should be monitored carefully and stopped if strong cytosolic background staining occurs, typically after 30 min using EGFP or 10 min using ECFP.

9. Wash samples 3 x 5 min in distilled water.
10. Post-fixation of samples in 1% osmium tetroxide reduced by 1.5% potassium ferrocyanide for 30 min on ice.
11. Wash samples 5 min in distilled water.
12. Dehydration by incubation in graded ethanol series of 50%, 70%, 80%, 90%, 95%, 3 x 100% 5 min each. The cells should not be allowed to dry out during this procedure nor at any other step.
13. Apply Epon 812 (Serva) on the cells for 1 h at room temperature.
14. Remove Epon by dripping off. Take care not to damage or dry the samples. Apply fresh Epon. Put a small Eppendorff tube without lid and filled with Epon on top of the cells. Once polymerized, it will serve as a block for mounting on ultramicrotome.
15. Polymerize samples at 60°C overnight.

Day 3: electron microscopy
After polymerization, the glass bottom of the culture dish is removed by immersion in concentrated hydrofluoric acid (30% HF) (Do not use glass containers for this!). Alternatively, the glass bottom can be detached by harsh temperature changes (liquid nitrogen –196°C). Control under a binocular microscope that the glass has been removed completely as any remnants will damage the diamond knife. In the binocular microscope, the bleached spots containing the cells of interest should be visible. If the glass had surface
marks (CellLocate or scratched by diamond), this will be visible as relief on the surface of the Epon block. Trim the resin block with a trimmer (Leica EM Trim) at angle of 30° to produce a pyramid containing the bleached cells. Ultrathin sections are cut parallel to the surface of the former coverslip on an ultramicrotome (Leica Ultracut S). The approach of the diamond knife edge to the sample should be done with care and as parallel as possible, to get the first sections already from the complete block. As flat embedded cells are only 5 – 20 μm in thickness, there is only limited material available for smoothing the block surface. Ultrathin sections of 40 – 70 nm thickness are collected on the surface of the knife water bath and mounted on Formvar coated grids. After drying for 1 h, the grids are ready for electron microscope analysis. Occasionally, sections can be counterstained with 1% aqueous uranyl acetate and lead citrate. But as these salts seem to have a higher affinity to osmicated compounds than to DAB precipitates, the signal-to noise ratio will be reduced. Therefore this is not recommended. Electron microscopy should be carried out using small apertures to enhance the contrast. Imaging with a sensitive digital camera will be advantageous. Semi-thin sections (100–250 nm) can also be analyzed by electron tomography, if the necessary equipment (electron microscope capable of recording well aligned tilt series, 3 D reconstruction software) is available.