Journal of Visualized Experiments

Visualization of endoplasmic reticulum subdomains in cultured cells

--Manuscript Draft--

Manuscript Number:	JoVE50985R1
Full Title:	Visualization of endoplasmic reticulum subdomains in cultured cells
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	endoplasmic reticulum; Fluorescent Proteins; Confocal microscopy; Fluorescence Recovery After Photobleaching; Fluorescence Loss In Photobleaching; ultrastructure; Transmission Electron Microscopy
Manuscript Classifications:	1.11.284.430.214.190.875.248: Endoplasmic Reticulum; 4.12.776.532.265: Green Fluorescent Proteins (GFP); 5.1.370.350.515.395: Microscopy, Confocal; 5.1.370.350.515.402.580: Microscopy, Electron, Transmission (TEM); 5.5.196.712.516.600.393: Fluorescence Recovery After Photobleaching (FRAP); 8.1.158.100.433: Cell Biology
Corresponding Author:	Maura Francolini, Ph.D. Fondazione Filarete - University of Milan Milano, ITALY
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	m.francolini@in.cnr.it;maura.francolini@unimi.it;maura.francolini@fondazionefilarete.com
Corresponding Author's Institution:	Fondazione Filarete - University of Milan
Corresponding Author's Secondary Institution:	
First Author:	Matteo Fossati, Ph.D.
First Author Secondary Information:	
Other Authors:	Matteo Fossati, Ph.D.
	Nica Borgese, Ph.D.
	Sara Francesca Colombo, Ph.D.
Order of Authors Secondary Information:	
Abstract:	The lipids and proteins in eukaryotic cells are continuously exchanged between cell compartments, although these retain their distinctive composition and functions despite the intense inter-organelle molecular traffic. The techniques described in this paper are powerful means of studying protein and lipid mobility and trafficking in vivo and in their physiological environment. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are widely used live-cell imaging techniques for studying intracellular trafficking through the exo-endocytic pathway, the continuity between organelles or sub-compartments, the formation of protein complexes, and protein localisation in lipid microdomains, all of which can be observed under physiological and pathological conditions. The limitations of these approaches are mainly due to the use of fluorescent fusion proteins, and their potential drawbacks include artifactual over-expression in cells and the possibility of differences in the folding and localisation of tagged and native proteins. Finally, as the limit of resolution of optical microscopy (about 200 nm) does not allow investigation of the fine structure of the ER or the specific sub-compartments that can originate in cells under stress (i.e. hypoxia, drug administration, the over-expression of transmembrane ER resident proteins) or under pathological conditions, we combine live-cell imaging of cultured transfected cells with ultrastructural analyses based on transmission electron microscopy.
Author Comments:	

Additional Information:	
Question	Response

.

DIPARTIMENTO DI BIOTECNOLOGIE
MEDICHE E MEDICINA TRASLAZIONALE

Maura Francolini
Dipartimento di Biotecnologie Mediche
e Medicina Traslazionale
Via Vanvitelli 32, 20129 Milano
Tel. 0250316977
maura.francolini@unimi.it

Milano, 21 May 2013

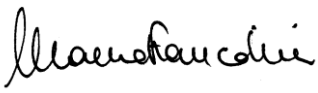
Dear Editor,

Enclosed you will find our revised manuscript entitled "**Visualization of endoplasmic reticulum subdomains in cultured cells**" by M. Fossati, N. Borgese S. Colombo, and myself (JoVE50985). As indicated in the submission site we would like the article to be filmed by the end of October 2013 as the first author and the main character of the movie will leave the laboratory. We hope that you will now consider the manuscript suitable for publication.

Thank you in advance for your consideration and please do not hesitate to contact us if you have any further question.

Looking forward to hearing from you,

Sincerely yours,



Maura Francolini

Visualisation of endoplasmic reticulum sub-domains in cultured cells

Authors:

Matteo FOSSATI[1,2,3]
m.fossati@in.cnr.it

Nica BORGESE[2,3,4]
n.borgese@in.cnr.it

Sara Francesca COLOMBO[2,3]
s.colombo@in.cnr.it

Maura FRANCOLINI[1,2]
maura.francolini@fondazionefilarete.com

[1]Fondazione Filarete, Milan, Italy
[2]Dept. of Medical Biotechnology and Translational Medicine, University of Milan, Italy
[3]Institute of Neuroscience, National Research Council (CNR), Milan, Italy
[4]Department of Health Science, "Magna Graecia" University of Catanzaro, Roccelletta di Borgia, Cosenza, Italy

Corresponding author:
Maura FRANCOLINI
Phone: +39 02 56660168/+39 02 50316977

Key words:
Endoplasmic reticulum (ER); fluorescent proteins (FPs); confocal microscopy; fluorescence recovery after photobleaching (FRAP); fluorescence loss in photobleaching (FLIP); ultrastructure; transmission electron microscopy (TEM),

SHORT ABSTRACT (50 words maximum)
We describe the imaging approaches we use to investigate the distribution and mobility of the transfected fluorescent proteins resident in the endoplasmic reticulum (ER) by means of the confocal imaging of living cells. We also ultrastructurally analyse the effect of their expression on the architecture of this subcellular compartment.

LONG ABSTRACT (150 words minimum, 300 words maximum)
The lipids and proteins in eukaryotic cells are continuously exchanged between cell compartments, although these retain their distinctive composition and functions despite the intense inter-organelle molecular traffic. The techniques described in this paper are powerful means of studying protein and lipid mobility and trafficking *in vivo* and in their physiological environment. Fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) are widely used live-cell imaging techniques for studying intracellular trafficking through the exo-endocytic pathway, the continuity between organelles or sub-compartments, the formation of protein complexes, and protein localisation in lipid microdomains, all of which can be observed under physiological and pathological conditions.

The limitations of these approaches are mainly due to the use of fluorescent fusion proteins, and their potential drawbacks include artifactual over-expression in cells and the possibility of differences in the folding and localisation of tagged and native proteins. Finally, as the limit of resolution of optical microscopy (about 200 nm) does not allow investigation of the fine structure of the ER or the specific sub-compartments that can originate in cells under stress (i.e. hypoxia, drug administration, the over-expression of transmembrane ER resident proteins) or under pathological conditions, we combine live-cell imaging of cultured transfected cells with ultrastructural analyses based on transmission electron microscopy.

INTRODUCTION

The discovery of green fluorescent protein (GFP) and its spectral variants, and the parallel development of fluorescence microscopy, have opened up completely new avenues for the investigation of protein behaviour in cells. Techniques such as fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP), which are possible because of the intrinsic capacity of fluorophores to extinguish their fluorescence under intense illumination, are based on confocal live-cell imaging and the use of transfected fluorescent fusion proteins[1-3]. They are widely used to assess not only the localisation of proteins, but also their mobility and vesicular transport, which can reveal important clues concerning their function[4].

The unique feature of eukaryotic cells is the presence of intracellular compartments that have specific lipid and protein compositions. Although organelles are physically isolated, they need to communicate with each other and share molecular components in order to maintain cellular homeostasis. The secretory pathway guarantees that the proteins and lipids synthesised in the ER reach the correct final destination in which they exert their function. Intracellular organelles can also be connected by dynamic contact sites that allow molecules (lipids) to be directly exchanged between compartments. Moreover, many proteins have to assembled in large heteromeric complexes or associated with specific lipid species (lipid rafts/microdomains) in order to become functionally active or to be transported to their final destination. All of these biological aspects greatly influence the kinetic properties of proteins, and can therefore be appropriately investigated by means of the techniques described below.

Our group has widely used FRAP and FLIP combined with electron microscopy in order to study the architecture of the ER and its different sub-domains. The ER is the first station of the secretory pathway and plays a key role in protein and lipid sorting[5]. It is a highly dynamic organelle whose distinct sub-domains reflect its many different functions (i.e. protein and lipid biosynthesis and trafficking, protein folding, Ca^{2+} storage and release, and xenobiotic metabolism). However, although they are morphologically, spatially and functionally distinct, these domains are continuous with each other, and their relative abundance can be modified in cells under physiological and pathological conditions. The best known and usually spatially segregated domains of the ER are the nuclear envelope, and the smooth and rough ER; however, we and others have demonstrated that there are ER structures with a more elaborate architecture and three-dimensional organisation in various cell types and tissues under physiological conditions that can also be induced by means of stressful stimuli such as hypoxia, drug administration or the over-expression of ER-resident transmembrane proteins[2,6] (and references therein).

We have also recently demonstrated the presence of such structures in cell models of human diseases[1,7]. Originating from the stacked cisternae of smooth ER, they were given the collective name of organised smooth endoplasmic reticulum (OSER) in 2003[6], although they are also known as *karmellae*, *lamellae* and crystalloid ER on the basis of their architecture

which, like their size, can vary. After the cells are transfected with GFP fused to the cytosolic region of tail-anchored (TA) ER-resident proteins (*d*EGFP-ER), the weakly dimerisng tendency of GFP in *trans* dramatically alters the organisation and structure of the ER. FRAP and FLIP experiments showed that *d*EGFP-ER is free to diffuse within OSERs, and the fact that it moves from the reticular ER to the OSER and *vice versa* indicates that the aggregates are continuous with the surrounding reticular ER. Ultrastructural analysis has allowed us to correlate the fluorescence data with a detailed description of OSER architecture and organisation at nanoscale level: OSERs are always made up of stacks of paired cisternae of smooth ER but may have different forms of spatial organisation, such as regularly arranged sinusoidal arrays or whorls, or hexagonal "crystalloid" tubular arrays. These rearrangements lead to cubic morphologies[8] which, as they have been found in cells under physiological conditions[9] and following stresses such as hypoxia[10], drug treatment[11] and cancer[9], may have significant potential as ultrastructural markers.

After this first demonstration using GFP fusion proteins, we used imaging experiments to analyse the proliferation of ER domains in response to pharmacological treatments[12], assess the tendency of fluorescent proteins to oligomerise in cells[13], and to investigate the role of a mutant, ALS-linked TA protein in the formation of intracellular aggregates of ER origin that may be relevant to its pathogenicity[1,8]. It has been suggested that the formation of intracellular aggregates (which occurs in many neurodegenerative diseases[14]) may be a protective mechanism designed to prevent the interactions between toxic mutant proteins and the surrounding cell components[15].

What follows is a description of a combination of optical and electron microscopy methods for investigating constructs whose C-terminal hydrophobic domains are inserted into the membrane of the ER, and an analysis of their dynamic behaviour and the effects of their over-expression on ER domain architecture in cultured cells (see Fig. 1 for a flowchart of the experimental protocol).

PROTOCOLS

1) Plasmid, cell culture and transfection with ER fluorescent proteins

1.1) The plasmid used in this study consists of an enhanced version of GFP fused at its C-terminus to the tail region of the ER isoform of rat cytochrome b(5) (abbreviated here as b(5)) via a linker sequence. The tail region contains the entire sequence (Pro94-Asp134) that remains membrane associated after the trypsin cleavage of native b(5), including the 17-residue TMD (Transmembrane domain), flanked by upstream and downstream polar sequences (UPS and DPS). The linker consists of the myc epitope followed by $[(Gly)_4Ser]_3$, and the entire cDNA is inserted into the Hind3-Xba1 sites of the mammalian expression vector pCDNA3. The details of the construction of this plasmid have been described in a previous publication in which it is referred to as GFP-ER[16].

1.2) Grow COS-7 cells in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1% penicillin/streptomycin in an incubator at 37°C and 10% CO_2.

1.3) Transfection. Plate $3x10^5$ cells on a round glass coverslip in a 6-well plate and, on the following day, transfect with the JetPEI system as described by the manufacturer. Note that the optimal JetPEI/DNA ratio has been tested in order to establish the maximum transfection efficiency depending on the plasmid and cell line used: in our case, a JetPEI:DNA ratio of 2:1 leads to 70-80% transfection efficiency.

2) Live fluorescence scanning confocal microscopy

2.1.) Live-cell imaging. Put the coverslip on which the transfected cells were seeded into a steel culture cell chamber for 24 mm coverslips filled with DMEM w/o phenol red, supplemented with 10% FBS, 2 mM L-glutamine, 1% pen/strep, 25 mM Hepes, 50 μg/ml cycloheximide and 1:100 OxyFluor to prevent the samples from photobleaching. An SP5 confocal microscope equipped with a temperature-controlled CO_2 incubator (37°C and 5% CO_2) is used for live cell imaging experiments, with *d*GFP-ER being visualised using a 488 nm laser and a 525/50 band pass emission filter.

2.2.) Fluorescence recovery after photobleaching (FRAP). Draw a region of interest (ROI), corresponding to an OSER structure, and bleach it using 20 iterations and a combination of 488 nm (100% of a 30 mW Argon laser, corresponding to 5.5-6 μW at the sample) and 405 nm (60% of a 30 mW Diode 405 laser, corresponding to 11.6 μW at the sample) lasers which, in our experience, leads to efficient and rapid photobleaching.

2.3) Record the recovery of fluorescence in the bleached ROIs by taking a single frame every 10 seconds for 10 minutes (pixel time = 1.61 μs/px).

2.4.) Fluorescence loss in photobleaching (FLIP). Draw a ROI corresponding to an OSER structure, and bleach as described above. The bleaching is repeated every 30 sec, and post-bleaching images are recorded every 10 seconds for 30 minutes (pixel time = 1.61 μs/px).

2.5) FRAP and FLIP analysis. All of the images are analysed using ImageJ software (http://rsbweb.nih.gov/ij/download.html). In the FRAP experiments, the fluorescence recovery of the bleached ROI is measured over time and normalised to the total fluorescence of bleached cell, which is always checked to be constant over time.

2.6) For the FLIP experiments, draw an ROI outside the bleached OSER and covering the whole cell. Measure its fluorescence intensity over time and normalise to the fluorescence levels of an ROI drawn on an unbleached cell in order to correct for any decrease in fluorescence caused by the imaging itself.

2.7) In all of the experiments, subtracted the background signal (determined in an area outside the cells) from the fluorescent intensities of the ROIs. Finally, plot the results using GraphPad Prism software.

3) Ultrastructural analysis by means of transmission electron microscopy

Given the toxicity of many of the reagents, all of the procedures should be carried out wearing an appropriate lab. coat and gloves under a fume hood.

3.1) After removing the coverslip from the Petri dish, fix the remaining cells grown on the bottom of the dish as a monolayer using filtered 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 10 min at room temperature.

3.2) Scrape the cells using a Teflon scraper and transfer them into 1.5 ml eppendorf tubes. Pellet the cells by means of centrifugation atat 9,000 g for 10 min. Remove the supernatant, add fresh fixative and leave overnight at 4°C.

3.3) Wash the pellets with the buffer, then post-fix with a solution of 1% osmium tetroxide in cacodylate buffer for 1 hour at room temperature.

3.4) Rinse with MilliQ water, and *en bloc* stain with 1% uranyl acetate in distilled water for between 20 min and 1 h.

3.5) Dehydrate the samples in increasing ethanol series (70%, 80%, 90%, 100% and 100% for 10 min each), and wash briefly twice in propylene oxide (15 mins each).

3.6) Infiltrate the samples in a mixture of propylene oxide + Epon (1:1) (from 2 h to overnight).
3.7) Embed in Epon epoxy resin cured at 60°C for at least 24 hours.

3.8) Section the manually trimmed resin blocs using an ultramicrotome LEICA UC6 equipped with a 45° diamond knife to obtain sections with a thickness of 60-70 nm. Collect the sections on 300 mesh copper grids.

3.8) Stain the sections on the grid with a saturated solution of uranyl acetate (20 min) and lead citrate (7 min), thoroughly wash the grids by immersing them in bi-distilled filtered water, and allow them to dry at room temperature.

3.9) The stained grids are observed using a TECNAI G2 transmission electron microscope, and the images are captured using a bottom-mounted CCD camera at different final magnifications (generally ranging from 6,000 to 39,000x).

REPRESENTATIVE RESULTS

Figure 2 shows an example FRAP study of protein mobility. The mobility of *d*EGFP-ER protein is demonstrated by the rapid fluorescence recovery after photobleaching in bleached OSERs. For the quantitative analysis, the half time and mobile fraction were derived from experimentally measured data by fitting the following monoexponential equation:

$$F(t) = F_{post} + \left(F_{rec} - F_{post}\right)(1 - e^{-\frac{t}{\tau}})$$

where F_{post} is the fluorescence signal after photobleaching, F_{rec} is the maximum fluorescence recovery value that is reach after bleaching, t the final time of registration and τ the mid time of registration.

Please note the importance of acquiring images without saturated pixels that could alter the fluorescence recovery and, consequently, the protein mobility analysis. It is also essential to always normalize the fluorescence signal in the bleached ROI to the total fluorescence of the same cell in order to consider fluorescence intensity variations due to bleaching during image acquisition or small changes in the focus plane.

An example of a FLIP experiment to study the continuity between intracellular compartments is shown in Fig. 3. OSERs are physically connected with the rest of the ER as demonstrated by the progressive emptying of the ER when the OSER domain is continuously bleached.

For a proper analysis, the acquisition of saturated pixels must be avoided (see above); furthermore, the acquisition parameters must be set up with laser powers as low as possible in order to avoid photobleaching due to image acquisition. For this reason it is strongly recommended to image an unbleached cell in the same field that will be used to normalize the fluorescence signal of the bleached cell.
All experiments have to be performed in the presence of cycloheximide, a translation inhibitor, in order to avoid any increase in the ER fluorescence signal (and consequently total fluorescence) due to protein biosynthesis.
Transmission electron microscopy demonstrated that the fluorescent aggregates observed in cultured cells transfected with *d*EGFP-ER represent patches of smooth and flattened ER cisternae that spatially organised themselves into well-defined 3D geometries classified on nthe basis of their patterns: linear or curved stacks (often associated with the nuclear envelope, not shown) (Figs. 4A and B) that may be continuous with regions of sinusoidal ER (Fig.4A); the membranes in some regions are organised into lattices with a square or hexagonal symmetry (crystalloid ER, not shown). Adjacent cisternae are separated by a thin layer of slightly electron-dense cytoplasm about 11 nm thick that is continuous with the cytoplasm surrounding the aggregates.

TABLES AND FIGURES

Figure 1: Flow chart of the experimental procedure. The cultured cells are first transfected with jetPEI (see Protocol) in order to over-express the fluorescent fusion protein of interest. After 24 h, the live transfected cells are visualised and FRAP and FLIP experiments are performed using a confocal microscope equipped with a controlled temperature CO_2 incubator, and the recorded images are exported and analysed using appropriate software (e.g. ImageJ). For the ultrastructural analysis, the transfected cells are fixed, pelleted and embedded in epon epoxy resin blocs. Ultrathin sections are obtained using a diamond knife, collected on copper grids, and observed under a transmission electron microscope.

Figure 2: FRAP experiment using COS-7 cells transiently transfected with *d*EGFP-ER. A) Two OSER structures (red ROIs) were bleached, and fluorescence recovery was recorded over time. Clear fluorescence recovery can be detected 1 min post-bleaching, and the signal further increases 4 min later (scale bar 10 μm). B): Quantitative analysis of the FRAP experiment showing the recovery half-time and the mobile fraction of the *d*EGFP-ER protein.

Figure 3: FLIP experiment using COS-7 cells transiently transfected with *d*EGFP-ER. A) The continuous bleaching of an OSER (indicated by the red ROI) causes a progressive decrease in fluorescence in the rest of the ER and in other OSER structures within the same cell (indicated by the yellow ROI). The yellow arrow indicates a portion of an unbleached cell in which the fluorescence signal is constant over time. (Scale bar 10 μm). B) Quantitative analysis of the FLIP experiment.

Figure 4: After fixation and embedding, cells expressing high levels of *d*EGFP-ER in which OSER structures could be detected by means of fluorescence optical microscopy were observed through a transmission electron microscope. A) Low magnification view of a portion of cytoplasm of a cell containing an OSER consisting of stacked cisternae and undulating sinusoidal membranes. Mitochondria (M) can be seen clustered around the OSER structures,

wheraes ribosomes decorate only the membranes of the outermost cisternae (arrowheads and inset). The 11 nm thick electron-dense space between the membranes is continuous with the cytoplasm (arrow and inset) (L = lysosomes/(auto-)phagosomes) (scale bar 1.5 μm; inset 0.25 μm). B) An OSER can be formed by lamellar ER: i.e. stacks of flattened ER cisternae that can be continuous or fragmented in their appearance in thin sections. Vesicles budding from the outermost cisternae of the stack can occasionally be observed (asterisk) (PM, plasma membrane) (Scale bar 150 nm).

DISCUSSION

The protocols and imaging approaches described in this paper have been used to investigate the distribution and mobility of transfected TA fluorescent proteins resident in the ER of living cells. We have also analysed the effect of the over-expression of these proteins on the architecture of this sub-cellular compartment by means of ultrastructural analyses.

The combination of live-cell confocal imaging and electron microscopy represents is a very powerful means of investigating the dynamic properties of proteins, and may provide important information concerning protein function. The described methods are not time consuming (typically three days of work), and the development of many user-friendly software applications for image acquisition and analysis makes photobleaching-based, live-cell imaging relatively simple.

The main limitation of these techniques is the use of fluorescent fusion proteins because the fluorescent tag can affect the proper folding and/or assembly of the protein of interest. In addition, over-expression can alter the behaviour of transfected, fluorescently tagged proteins, and may therefore not reflect the real properties of endogenous proteins; however, this can be overcome by using of inducible and stably transfected cells in which the expression level can be precisely modulated to obtain levels comparable with those of the endogenous protein[1,7]. The tendency of FPs to oligomerise has been widely documented and could significantly alter the behaviour (i.e. kinetics, unwanted protein-protein interactions and formation of aggregates) of chimeric proteins. The use of optimised monomeric fluorescent proteins should therefore be considered[17].

Another critical aspect of dynamic imaging studies using fluorescence and photobleaching is the time needed to bleach the fluorescence efficiently and measure fluorescence recovery (and thus protein mobility) precisely, which also depends on the area of the ROI and local cell thickness. If a given GFP-tagged protein has a high diffusion coefficient, diffusion could occur during the bleaching and thus interfere with recovery time measurements. In order to obtain rapid and efficient bleaching, it is strongly recommended that a “zoom in” function (if available) and more than one laser line be used. Although the use of a fast scan module (i.e. a resonant scanner) can greatly improve the speed of imaging during the recovery phase of an experiment, in our hands it also considerably reduces bleaching efficiency. However, alternative scanning systems (such as a spinning disc equipped with a dedicated photobleaching device), and more powerful lasers can improve both bleaching efficiency and acquisition speed.

Most fluorescent proteins used in FRAP and FLIP experiments show some degree of reversible photobleaching and blinking that must be considered when performing quantitative analyses. The fluctuations between fluorescent and dark states occur in the second to minute time-scale. For EGFP, It has been shown that during bleaching experiments, fluorescence variations might involve less than 10% of the molecules, thus in the present protocol this phenomenon is negligible. If all conditions are kept constant, this will introduce a constant bias in the results. If other fluorescent proteins are used, in which the reversible fraction is

significantly higher (i.e. YFP), or to detect and evaluate photobleaching reversibility, this can be done by measuring fluorescence recovery after photobleaching in the entire living cell; if recovery is observed this can only be the result of photobleaching reversibility[18].

The potential toxicity of light during the experiments is another critical factor, particularly because photobleaching requires strong lighting. It is well known that excited fluorophores tend to react with oxygen to produce free radicals that can affect various intracellular processes and even cell viability[19], and so it is necessary to establish a balance between efficient bleaching and minimal phototoxicity; furthermore, cell viability should always be checked after live-cell imaging experiments. Given the short recording time, we did not consider the genotoxic effect of short wavelength light (405 nm) in the example described in this paper but, if a longer experiment is needed, a 405 nm laser line should not be used.

We chose not to use a correlative approach to transmission electron microscopy because of the heterogeneous nature of OSER architecture and the fact that we wanted to observe as many cells (and structures) as possible. The diversity of the fine structure of protein aggregates in cells may be a key feature of different diseases and we were interested in obtaining a broad range of samples, whereas a correlative approach allows the observation of fewer events during the same period time. However, correlative light-electron microscopy (CLEM) should be the first choice when investigating events in structures that cannot be easily identified (such as less prominent ER sub-domains) or in a limited number of cells (such as micro-injected cells). It is worth noting that our experiments were characterised by a high degree of transfection efficiency (at least 30% of the cells were transfected), otherwise the possibility of observing OSER structures non-correlatively is quite limited.

Disclosure

The authors have nothing to disclose.

Acknowledgements

The authors are grateful to Fondazione Filarete for its help and support in the publication of this article. We would also like to thank Centro Europeo di Nanomedicina for the use of the TECNAI G2 transmission electron microscope.

REFERENCES

1 Fasana, E. *et al.* A VAPB mutant linked to amyotrophic lateral sclerosis generates a novel form of organized smooth endoplasmic reticulum. *Faseb J* **24**, 1419-1430 (2010).

2 Borgese, N., Francolini, M. & Snapp, E. Endoplasmic reticulum architecture: structures in flux. *Curr Opin Cell Biol* **18**, 358-364 (2006).

3 Ronchi, P., Colombo, S., Francolini, M. & Borgese, N. Transmembrane domain-dependent partitioning of membrane proteins within the endoplasmic reticulum. *J Cell Biol* **181**, 105-118 (2008).

4 Lippincott-Schwartz, J., Snapp, E. & Kenworthy, A. Studying protein dynamics in living cells. *Nature Rev. Mol. Cell Biol.* **2**, 444-456 (2001).

5 Lee, M. C., Miller, E. A., Goldberg, J., Orci, L. & Schekman, R. Bi-directional protein transport between the ER and Golgi. *Annu Rev Cell Dev Biol* **20**, 87-123 (2004).

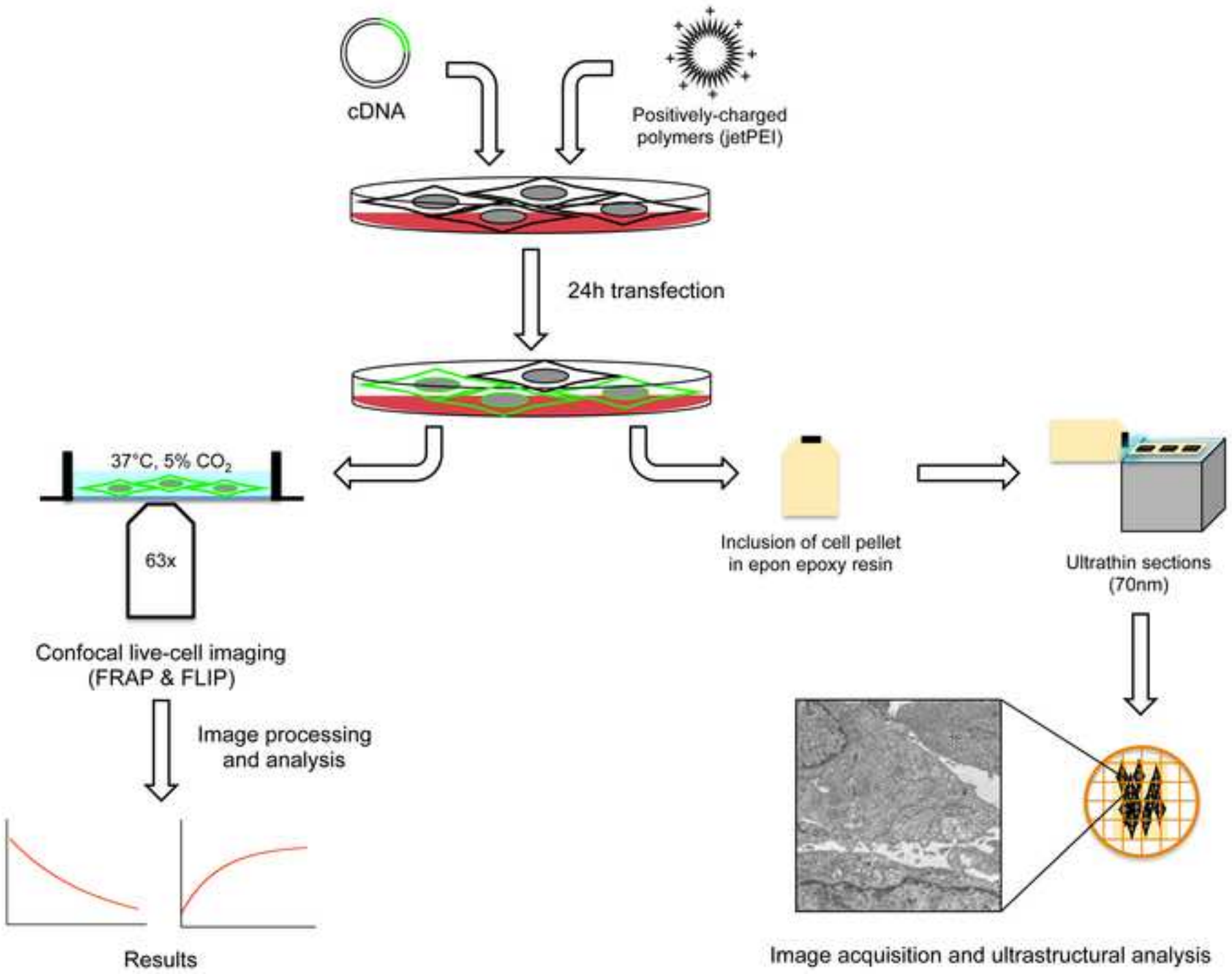
6 Snapp, E. L. *et al.* Formation of stacked ER cisternae by low affinity protein interactions. *J. Cell Biol.* **163**, 257-269 (2003).

7 Papiani, G. *et al.* Restructured endoplasmic reticulum generated by mutant amyotrophic lateral sclerosis-linked VAPB is cleared by the proteasome. *Journal of cell science* **125**, 3601-3611, doi:10.1242/jcs.102137 (2012).

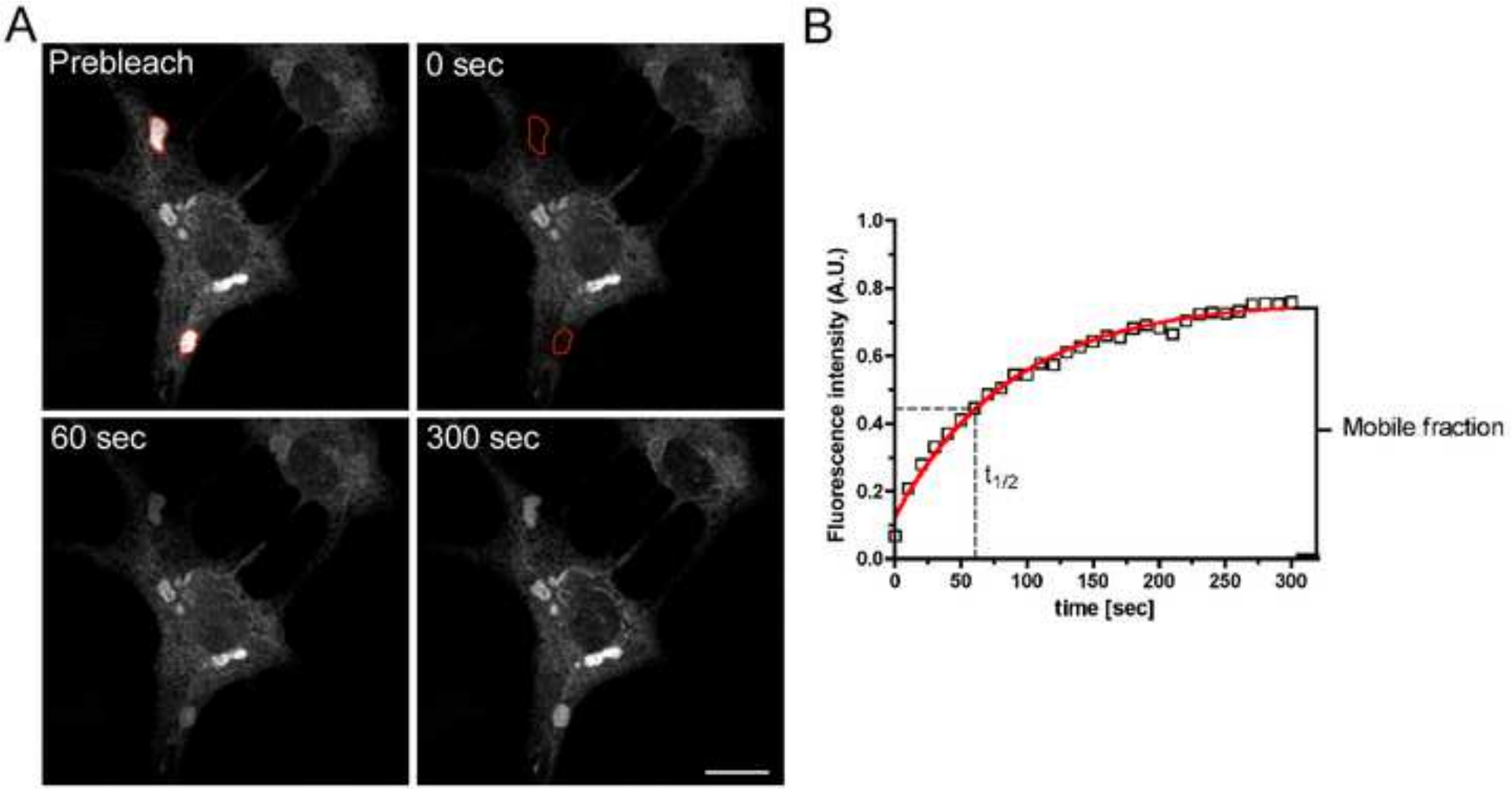
8 Almsherqi, Z. A., Kohlwein, S. D. & Deng, Y. Cubic membranes: a legend beyond the Flatland* of cell membrane organization. *The Journal of cell biology* **173**, 839-844, doi:10.1083/jcb.200603055 (2006).
9 Federovitch, C. M., Ron, D. & Hampton, R. Y. The dynamic ER: experimental approaches and current questions. *Current opinion in cell biology* **17**, 409-414, doi:10.1016/j.ceb.2005.06.010 (2005).
10 Takei, K., Mignery, G. A., Mugnaini, E., Sudhof, T. C. & De Camilli, P. Inositol 1,4,5-trisphosphate receptor causes formation of ER cisternal stacks in transfected fibroblasts and in cerebellar Purkinje cells. *Neuron* **12**, 327-342 (1994).
11 Feldman, D., Swarm, R. L. & Becker, J. Ultrastructural study of rat liver and liver neoplasms after long-term treatment with phenobarbital. *Cancer research* **41**, 2151-2162 (1981).
12 Sprocati, T., Ronchi, P., Raimondi, A., Francolini, M. & Borgese, N. Dynamic and reversible restructuring of the endoplasmic reticulum induced by PDMP in cultured cells. *J. Cell Sci* **119**, 3249-3260 (2006).
13 Pyszniak, A. M., Welder, C. A. & Takei, F. Cell surface distribution of high-avidity LFA-1 detected by soluble ICAM-1-coated microspheres. *Journal of immunology* **152**, 5241-5249 (1994).
14 Taylor, J. P., Hardy, J. & Fischbeck, K. H. Toxic proteins in neurodegenerative disease. *Science* **296**, 1991-1995 (2002).
15 Winklhofer, K. F., Tatzelt, J. & Haass, C. The two faces of protein misfolding: gain- and loss-of-function in neurodegenerative diseases. *The EMBO journal* **27**, 336-349, doi:10.1038/sj.emboj.7601930 (2008).
16 Borgese, N., Gazzoni, I., Barberi, M., Colombo, S. & Pedrazzini, E. Targeting of a tail-anchored protein to endoplasmic reticulum and mitochondrial outer membrane by independent but competing pathways. *Mol. Biol. Cell* **12**, 2482-2496 (2001).
17 Chudakov, D. M., Matz, M. V., Lukyanov, S. & Lukyanov, K. A. Fluorescent proteins and their applications in imaging living cells and tissues. *Physiol Rev* **90**, 1103-1163, doi:10.1152/physrev.00038.2009 (2010).
18 Bancaud, A., Huet, S., Rabut, G. & Ellenberg, J. Fluorescence perturbation techniques to study mobility and molecular dynamics of proteins in live cells: FRAP, photoactivation, photoconversion, and FLIP. *Cold Spring Harb Protoc* **2010**, pdb top90, doi:10.1101/pdb.top90 (2010).
19 Michida, T. *et al.* Role of endothelin 1 in hemorrhagic shock-induced gastric mucosal injury in rats. *Gastroenterology* **106**, 988-993 (1994).

1

Click here to download high resolution image

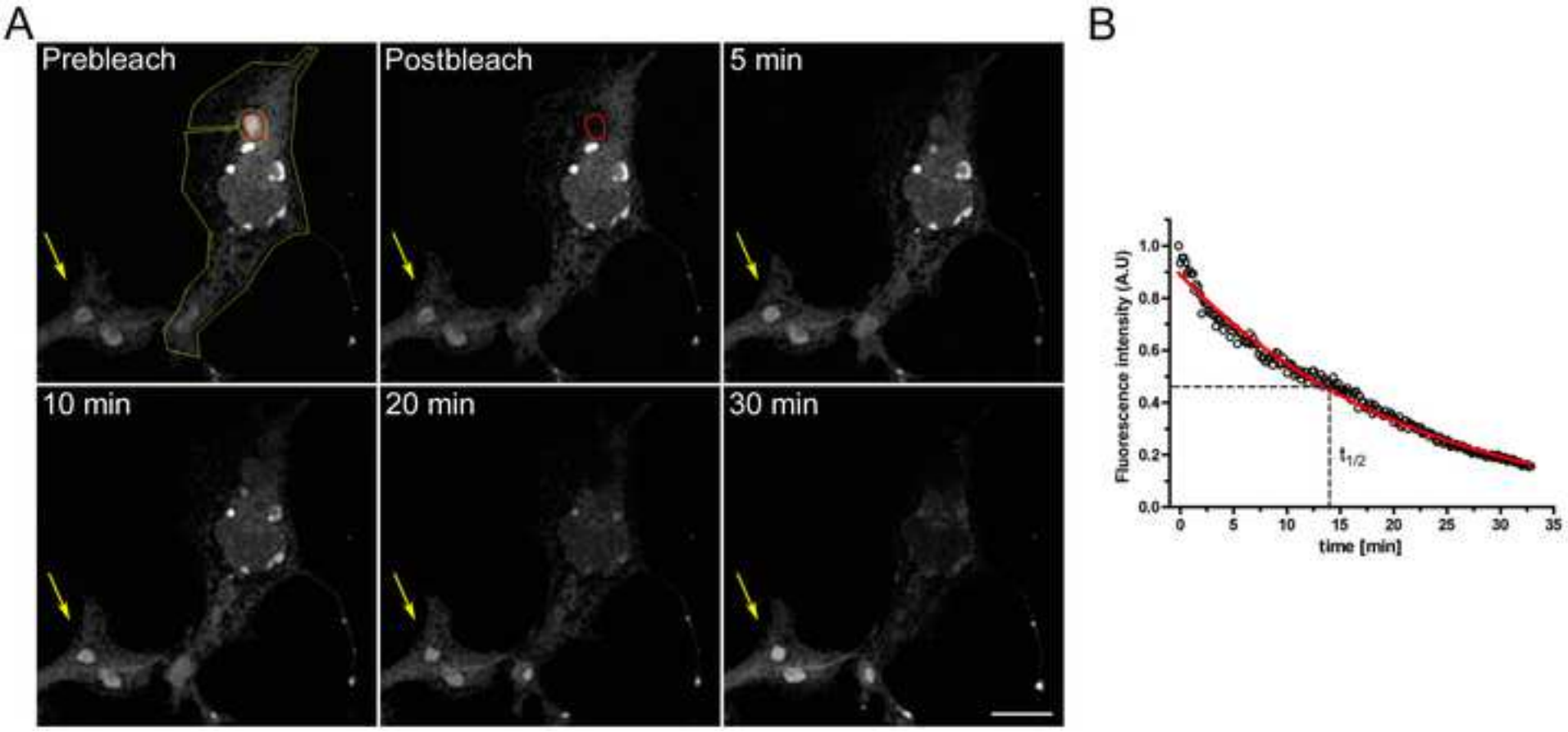


2
Click here to download high resolution image

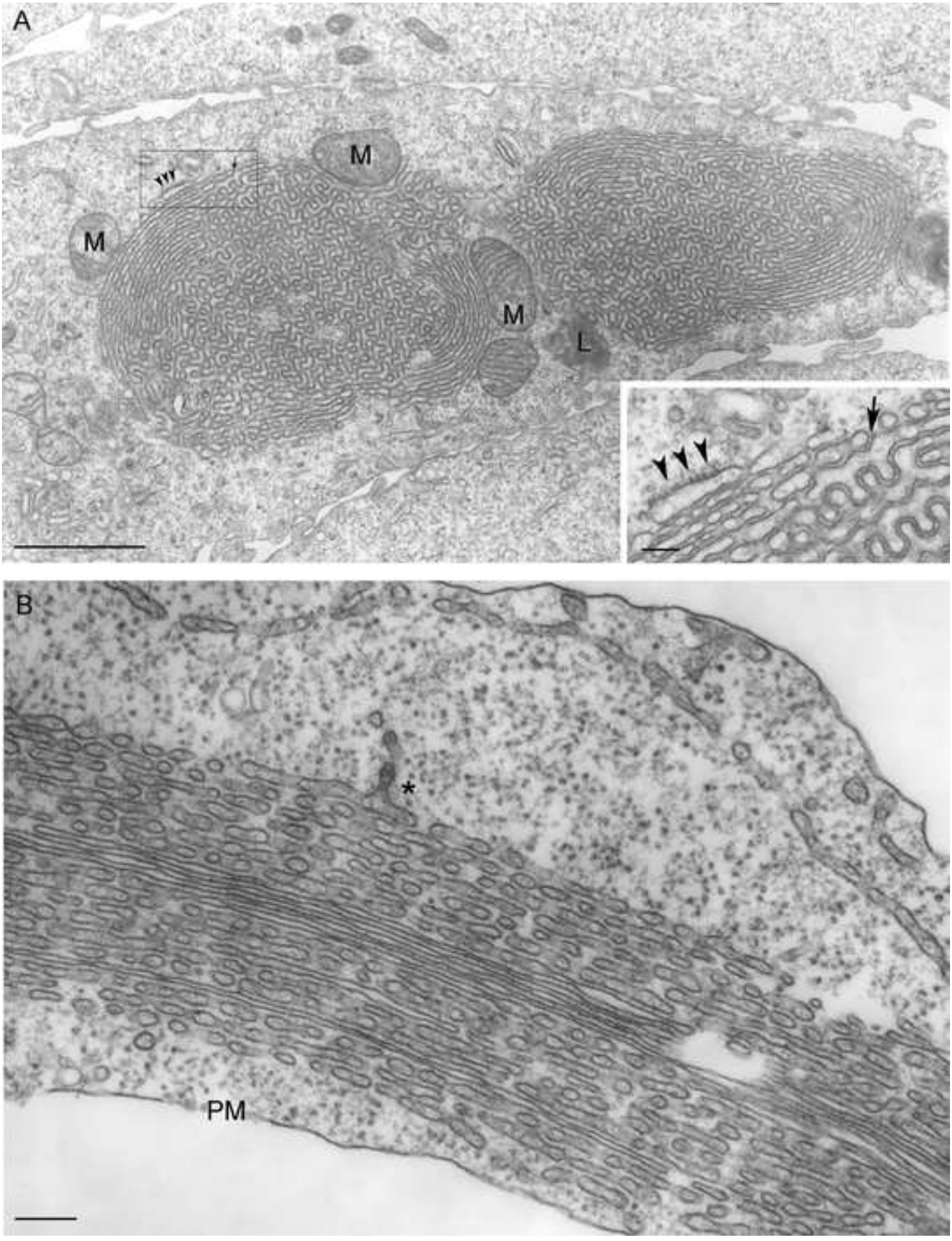


3

Click here to download high resolution image



Click here to download high resolution image



*Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment: JoVE_Materials_Fossati et al., 2013 RE.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Dulbecco's Modified Eagle medium (DMEM)	Invitrogen	41966029	
Dulbecco's Modified Eagle medium (DMEM) w/o phenol red	Invitrogen	31053028	
Fetal Bovine Serum (FBS)	Invitrogen	10270106	
Pen/Strep	Invitrogen	15140-122	
L-Glutamine 200 mM solution	Invitrogen	25030-024	
jetPEI	Polyplus Transfection	PP10110	
OxyFluor	Oxyrase Inc.	OF-0005	
Glutaraldehyde Grade I	Sigma Aldrich	G5882	
Sodium Cacodylate Trihydrate	Sigma Aldrich	C0250	
Osmium Tetroxide 4% solution	Electron Microscopy Science	19150	
Uranyl Acetate dihydrate	Sigma Aldrich	73943	slightly radioactive
Propylene Oxide	Sigma-Aldrich	82320	
EPON embedding medium kit	Sigma-Aldrich	45359-1EA-F	
Lead Citrate	Electron Microscopy Science	17800	
Bench top centrifuge	Eppendorf	5415 D	
Spectral Confocal Microscope	Leica Microsystems	TCS SP5	
CO_2 Microscope Cage Incubation System	OkoLab		
Ultramicrotome	Leica Microsystems	UC6	
Diamond knife	Diatome	Ultra 45°	
Transmission Electron Microscope	FEI	Tecnai G2	
GraphPad Prism Software	GraphPad Software, Inc		
steel culture cell chamber for 24mm coverslip	Bioscience Tools	CSC-25	
Electron Microscopy grids	Electron Microscopy Science	G300Cu	

jove JOURNAL OF VISUALIZED EXPERIMENTS
17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: VISUALIZATION OF ENDOPLASMIC RETICULUM SUBDOMAINS IN CULTURED CELLS

Author(s): M. FOSSATI, N. BORGESE, S.F. COLOMBO, M. FRANCOLINI

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ☒ Standard Access ☐ Open Access

Item 2 (check one box):

☒ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the

Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including,

17 Sellers Street
Cambridge, MA 02139
tel. +1.617.945.9051
www.JoVE.com

ARTICLE AND VIDEO LICENSE AGREEMENT

without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

AUTHOR:

Name: MAURA FRANCOLINI

Department: FONDAZIONE FILARETE - MEDICAL BIOTEC. AND TRANSLATIONAL MEDICINE

Institution: UNIVERSITÀ DEGLI STUDI DI MILANO ITALIA

Article Title: VISUALIZATION OF ENDOPLASMIC RETICULUM SUBDOMAINS IN CULTURED CELLS

Signature: Maura Francolini Date: 25th March 2013

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy as a PDF to the *JoVE* submission site upon manuscript submission (preferred);
2) Fax the document to +1.866.381.2236; or
3) Mail the document to *JoVE* / Attn: *JoVE* Editorial / 17 Sellers St / Cambridge, MA 02139

For questions, please email editorial@jove.com or call +1.617.945.9051.

MS # (internal use):

The Authors are very grateful to the Reviewers for their thoughtful and stimulating reviews. A point-to-point response (in bold) to the criticisms is herewith provided.

Reviewer #1:

Major Concerns:
1) The manuscript reads as a hybrid between a purely methodological guide for studying ER subdomains and a "minireview" of OSERs. Related to this issue is that from the manuscript it is unclear whether the presented techniques are specific for the study of OSERs or that they would serve more generally for studying ER subdomains. If the latter is the objective of the authors, they may want to compare their practices on OSERs with approaches that have been employed elsewhere on ER (subdomains) with similar questions in mind, such as membrane fluidity and exchange of resident proteins between ER subdomains. In any case the "OSER angle" is an interesting example, but perhaps it is worthwhile to discuss in more detail how the authors think OSERs may reflect the physiology of the cell other than being a "freaky" phenomenon induced by over-expression by either ER-GFP or mutant VAPB.

In the "Introduction " we have added a paragraph in which we introduce studies by other groups describing the role and significance of ER membrane proliferation and more generally of intracellular membranes proliferation in physiological conditions and upon stressful stimuli (i.e. hypoxia, drug administration), disease (cancer) and in neurodegenerative disease where aggregates of mutant proteins (the causative agents of the disease itself) might represent a protection against unwanted and potentially harmful interaction of the mutants with the intracellular environment.

2) The EM part, while technically valid, doesn't really appear integrated with the live-cell imaging and doesn't add much beside the spectacular visualization of OSER structures. In the absence of a correlative analysis it is difficult to envision how these findings can serve in the analysis of other, less prominent ER subdomains etc. Perhaps, the authors can elaborate more comprehensively how EM should be employed to complement the FLIP and FRAP.

The importance of the investigation of the fine structure of subcellular aggregates and, more specifically in the present manuscript of these ER subcompartments, has been better explained in the "Introduction" and in the "Discussion" sections where we report the relevance of this approach in the identification of markers for ultrastructural pathology. Regarding the advantage of using a correlative microscopical approach in the study of less prominent ER subdomains, this has been fully acknowledge in the end of the "Discussion" section.

Minor Concerns:

1) I have interpreted that the authors refer to their constructs as ER resident, while this term is generally used for lumenal proteins. To avoid confusion I suggest to explicitly state in each case that it concerns tail anchored proteins in the ER membrane facing the cytosol or find a term that fits such a profile.

To avoid confusion, throughout the text, we have specified that all our experiments were performed using tail anchored (TA) resident ER proteins, although we do not completly agree about "resident" beeing specific for lumenal proteins.

2) The English can improve in some parts.

The new version of the manuscript was revised by a professional english translator

3) Ref 4: I believe Snappe should be Snapp.

The reference was corrected

Reviewer #2:

Minor Concerns:

1) The manuscript describes well how to prepare the sample, perform confocal imaging, and analyze the FRAP and FLIP measurements. However, to make the protocol less instrument-specific it would be convenient to report parameters as the laser power at the sample plane and the pixel dwell time, both during imaging and bleaching (see Additional Comments).

Additional Comments to Authors:
FRAP and FLIP: laser intensities for bleaching should be reported in mW, preferably at the sample, and not as "% of laser power", since this indication does not provide sufficient information about the actual energy delivered to the sample. Alternatively, if such a measure is not possible, measuring the laser power before the objective along with the transmission of the microscope objective at the wavelengths used for FRAP and FLIP would give a better idea about the laser intensity during the photobleaching experiments.
It is not clear how the fluorescence intensity for a FLIP experiment is measured: please specify in section 2.3 whether the fluorescence was integrated over the whole image or if a specific ROI of the cell was selected. In the latter case, please report in Figure 3 panel A the ROI over which the loss of fluorescence shown in panel B was measured.

Scanning speed (pixel time) has been indicated and laser intensities during bleaching have been reported as mW at the sample. In fact we measured the laser intensities both before and after the objective using a Plan Neofluar 20x/0.5 objective. Very little differences were recorded in the two measures (less than 1μW). The value at the sample were included in the manuscript. We have specified the nature of the ROI in the FLIP experiment and it has been reported in Fig. 3 Panel A,

Reviewer #3:

Major Concerns:

1)In order to compare the light microscopy level and the ultrastructure, CLEM (correlative light-electron microscopy) is considered the method of choice. The possibility of correlation between FRAP and FLIP behavior and the ultrastructure of the same OSERs would be a great advantage for the study. This point should be better discussed and the reason why they do not use CLEM should be better explained.

As reported earlier, we stated at the end of the "discussion" section that we performed a non-correlative EM approach because we wanted to investigate the fine structure of as many aggregates as possible in many different cells in a limited amount of time. These fluorescent aggregates were very broad and abundant in cells and as the transfection efficiency was quite high we had no problem in tracking the structure of interest in many different samples. We clearly stated that, in case of less prominent features to be investigated or in case of a low number of cell presenting the wanted phenotype (i.e. microinjected cells), a correlative approach must be the method of choice.

2) the paper is missing an organic discussion of the limitations of the methods, and in particular of FRAP and FLIP experiments. Only some potential pitfalls are mentioned, but spread throughout the manuscript. Furthermore, other issues that affect photobleaching experiments should be discussed in addition to the ones already considered:
a) FRAP kinetics could be substantially affected by the area of the ROI and the local cell thickness.
b)fluorochrome intermittency (bliking or dark state) could affect the recovery curves
c) photo-toxicity induced by the strong laser exposure could affect several cellular processes, such as vesicular transport.
Due to these limitations, the FRAP and FLIP experiments presented in the paper demonstrate the continuity between the OSERs and the rest of the ER, but are not suitable for the determination of kinetics and diffusion parameters. This point should be discussed.

As indicated by the reviewer we have expanded the description of the limitations of the methods we propose in the "discussion". In particular, as suggested, we mentioned the importance of the size of the ROI and the local cell thickness, while we had already indicated and discussed the fact that phototoxicity induced by high intensity irradiation can affect different intracellular processes and even viability.
A paragraph has been added in the "Discussion" to introduce the blinking behaviour of GFP and its potential influence on FRAP measurements, influence that is quite low due to the fact that 1) only a small fraction (less than 10%) of GFP molecules undergoes reversible photobleaching upon illumination and 2) if imaging conditions are kept constant, a constant bias will be introduced in the results.

3)the use of the 405 laser for bleaching in the FRAP and FLIP experiments is not justified. GFP is not significantly excited at this wavelength and photo-toxicity increases with shorter wavelengths. If there is a specific reason why they used this laser, it should be discussed.

We have described in the "Protocol" section of the manuscript the reason why we used 488 e 405 laser lines for bleaching, it is a common useful practice to use more than one laser line to increase the efficiency and the speed of bleaching. In the "Discussion" we discussed about the limitation of the use of the 405 laser line because of its genotoxic added to the more widespread phototoxicity of high intensity laser excitation.

Minor Concerns:
1) it is not clear what the authors mean by "round-shaped chamber" in paragraph 2.1. Manufacturer and cat number should be indicated as for the other equipment

The nature of the object has been clarified in the text and the manufacturer and cat. number indicated in the text and in the excel file.

2) EM grids are not listed in the table of reagents as well
The item has been included in the excel file.

3) references are poor (especially in the introduction) and mostly limited to the authors' publications

More references have been cited in the "Introduction" and in the "Discussion" sections.

4) english should be revised

As mentioned above this new version manuscript has been revised by a professional english translator.

Reviewer #4:

1. The authors may spend a couple of sentences in the discussion about the use of optimized monomeric fluorescent proteins (cf. Chudakov et al., Physiol Rev, 2010) to avoid unwanted protein-protein interactions or the formation of artefactual ER structures due to the mentioned oligomerization tendency of FPs.

A paragraph discussing this interesting and crucial aspect of the use of fluorescent proteins as tag has been added in the "Discussion". We are grateful to the reviewer for the suggestion.

2. There is an inconsistency between the text and figure 1. It is reported that cells were grown and imaged in 10% CO2 but the figure indicates a 5% concentration.

This inconsistency has been fixed in the revised version of the manuscript.

3. Please indicate the source of the softwares used for data analysis.

The source of the used softwares has been indicated in the text.