Imaging ER-to-Golgi transport: towards a systems view

Fatima Verissimo and Rainer Pepperkok
European Molecular Biology Laboratory, Cell Biology and Cell Biophysics Unit, Meyerhofstraße 1, 69117 Heidelberg, Germany
Authors for correspondence (verissim@embl.de; pepperko@embl.de)

Journal of Cell Science 126, 5091–5100
© 2013, Published by The Company of Biologists Ltd
doi: 10.1242/jcs.121061

Summary
Proteins synthesised at the endoplasmic reticulum (ER) have to undergo a number of consecutive and coordinated steps to reach the Golgi complex. To understand the dynamic complexity of ER-to-Golgi transport at the structural and molecular level, light microscopy approaches are fundamental tools that allow in vivo observations of protein dynamics and interactions of fluorescent proteins in living cells. Imaging protein and organelle dynamics close to the ultra-structural level became possible by combining light microscopy with electron microscopy analyses or super-resolution light microscopy methods. Besides, increasing evidence suggests that the early secretory pathway is tightly connected to other cellular processes, such as signal transduction, and quantitative information at the systems level is fundamental to achieve a comprehensive molecular understanding of these connections. High-throughput microscopy in fixed and living cells in combination with systematic perturbation of gene expression by, e.g. RNA interference, will open new avenues to gain such an understanding of the early secretory pathway at the systems level. In this Commentary, we first outline examples that revealed the dynamic organisation of ER-to-Golgi transport in living cells. Next, we discuss the use of advanced imaging methods in studying ER-to-Golgi transport and, finally, delineate the efforts in understanding ER-to-Golgi transport at the systems level.

Key words: Early secretory pathway, Endoplasmic reticulum, Golgi, Transport carriers, Microscopy

Introduction
The secretory pathway consists of a number of membrane-bound compartments, such as the endoplasmic reticulum (ER), the Golgi complex, and pleomorphic membrane carriers that transport proteins and lipids to their destination (Murshid and Presley, 2004). Remarkably, approximately one-third of all cellular proteins move through the secretory pathway to reach their final destination in the intra- or extracellular space (Dancourt and Barlowe, 2010), underlining the importance of this pathway for cell function. The current models that describe how material moves through the secretory pathway are grounded on fundamental electron microscopy (EM) observations made by Palade and colleagues (Jamieson and Palade, 1967; Jamieson and Palade, 1968). Based on this and related work, in 1975, Palade proposed a model suggesting that the path taken by secreted proteins occurs in vesicular-like transport carriers that move vectorially from the rough ER to the Golgi complex and, from there, to the plasma membrane (Palade, 1975).

Efficient ER-to-Golgi transport requires a number of coordinated steps (see Fig. 1). In Saccharomyces cerevisiae, secretory proteins that are synthesised at the ER are sorted and concentrated by the vesicular coat-protein complex COP II, and incorporated into COP II-coated transport vesicles that deliver their cargo to the Golgi complex (Lee et al., 2004; Rossanese et al., 1999). COP II-coated transport vesicles are then returning the transport machinery back to the ER (Lee et al., 2004). In mammalian cells, Drosophila melanogaster and some yeast (e.g. Pichia pastoris), COP II-coated transport carriers form only at specialised sites at the ER, the so-called ER-exit sites (ERES) (Bevis et al., 2002; Herpers and Rabouille, 2004; Stephens, 2003) (see also Fig. 1). In mammalian cells, COP II vesicles are thought to form vesicular tubular clusters (VTCs), a more complex transport intermediate, by homotypic fusion (see Fig. 1 for the mammalian transport model) (Hughes and Stephens, 2008). VTCs, subsequently acquire the vesicular coat complex COP I and then move along microtubules to the Golgi complex where they deliver their cargo. COP I-coated transport vesicles form at the Golgi complex to return misfolded proteins and the transport machinery back to the ER (Fig. 1). Alternative transport models suggest that COP I vesicles first deliver their cargo in a microtubule-independent manner to a more stable ER-to-Golgi intermediate compartment (ERGIC). Secretory cargo is then thought to move from the ERGIC towards the Golgi complex in VTC-like structures whose nature is not yet clear (see Fig. 1) (Appenzeller-Herzog and Hauri, 2006). Although most of the basic steps in ER-to-Golgi transport are similar in different species, there are variations regarding transport carriers or cytoskeleton structures involved, e.g. between plant cells and mammalian cells, that to date are not completely understood (Brandizzi and Barlowe, 2013).

The molecular core machinery that is responsible to drive the different transport steps in the early secretory pathway is conserved between species, and might be considered as being largely identified and fairly well characterised (Béthune et al., 2006; Brandizzi and Barlowe, 2013; Dancourt and Barlowe, 2010; Gürkan et al., 2006; Hsu and Yang, 2009; Lee et al., 2004; Lippincott-Schwartz and Liu, 2006; Szul and Sztul, 2011; Zanetti et al., 2012). However, the additional factors that regulate the different transport steps in the early secretory pathway in response to changes in the cellular environment or mediate alterations in the secretory pathway during the various phases in development or cell differentiation are still unknown. In vitro biochemical assays were used very successfully to identify and characterise the core machinery of the early secretory pathway (Barlowe et al., 1994;
Golgi complex

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>cis</td>
<td>trans</td>
<td>medial</td>
</tr>
</tbody>
</table>

Fig. 1. Illustration of ER-to-Golgi transport in mammalian cells. Shown are the ER-to-Golgi interface and the transport carriers involved. In mammalian cells, COPII vesicles bud from ribosome-free areas in the ER, the so-called ER-exit sites (ERES) (see also Fig. 2A). COPII-coated vesicles vary in size, but are typically between 60 and 80 nm in diameter. COPII components are directly involved in cargo selection and membrane bending and, therefore, essential for protein export from the ER. COPII vesicles undergo homotypic fusion to form vesicular tubular clusters (VTCs). Although forming VTCs might be partially coated with COPII and COPI (*), VTCs that move along microtubules in order to fuse and deliver cargo to the Golgi are COPI-coated. COPI-coated vesicles also retrieve components of the transport machinery as well as misfolded proteins from the Golgi and VTCs back to the ER. Typical COPI vesicles are 50–60 nm in size and the size of VTCs varies from 250 nm to 1 μm. There is some evidence to support the existence of a more stable ER-to-Golgi intermediate compartment (ERGIC); however, the nature of the carriers that move cargo from the ERGIC to the Golgi complex is not clear. TGN, trans-Golgi network. Arrows indicate the direction of transport carriers; see Table 1 for the roles of minimal COPII and COPI components.

Fries and Rothman, 1980; Lee et al., 2004; Rothman, 1996). However, these methods have limitations in achieving a full understanding at the whole-cell or organism level, as this requires analyses in the context of the whole cell or organism. We propose that one way to obtain systems-level information is by quantitative fluorescence microscopy in living cells. Although transport carriers in the early secretory pathway are typically smaller than the resolution of conventional light microscopy, recent technological progress—such as live cell imaging with high temporal and spatial resolution using fluorescently tagged proteins (FPs), the development of correlative light and electron microscopy approaches, as well as the availability of super-resolution light microscopy—has taken place. These technologies and high-throughput microscopy methods, together with the possibility to perturb gene expression by using RNA interference (RNAi) in mammalian cells at large scale, have opened new avenues to gain, indeed, an understanding of the secretory pathway at the systems level.

Here, we review and discuss distinct imaging approaches to study the early secretory pathway. We will discuss how they helped to gain a better understanding of membrane traffic at the ER-Golgi boundary during the past few years, including the latest developments in advanced imaging methods that have the potential to help understanding ER-to-Golgi transport at the systems level.

Imaging ER-to-Golgi transport in living cells

A major turning point for the field of membrane traffic was the availability of the green fluorescent protein (GFP) and its spectral variants (Crivat and Taraska, 2012; Giepmans et al., 2006; Shaner et al., 2005; Stepanenko et al., 2011). Fluorescent labelling and time-lapse microscopy provided the means to visualise almost any protein in its natural environment, and to monitor its localisation (Huh et al., 2003; O’Rourke et al., 2005; Simpson et al., 2000) and dynamics in living cells. Most remarkably, this approach revealed the highly dynamic nature of the organisation of ER-to-Golgi transport and the organelles involved (Brandizzi and Barlowe, 2013; Verissimo and Peppercok, 2008).

For example, time-lapse microscopy of fluorescently labelled COPII proteins allowed the visualization of how ERES change during the cell cycle; they disappear before metaphase and reappear towards the end of cell division (Stephens, 2003) (Fig. 2A; supplementary material Movie 1). Imaging of fluorescently labelled secretory cargo showed that VTCs, which are highly similar to the ER-to-Golgi intermediate compartment (ERGIC), are not static structures like the ERGIC (Appenzeller-Herzog and Hauri, 2006; Ben-Tekaya et al., 2005), but rather long-range transport carriers (Presley et al., 1997; Scales et al., 1997). Moreover, these cargo carriers form in the vicinity of COPII-labelled ERES and later segregate to acquire the vesicular coat complex COPI and move towards the Golgi complex in a microtubule-dependent manner (Shima et al., 1999; Stephens et al., 2000) (see also Fig. 1; Fig. 2B; supplementary material Movie 2).

Time-lapse microscopy and fluorescence recovery after photobleaching (FRAP) (see Box 1) of fluorescently tagged Golgi markers also revealed the highly dynamic nature of this organelle (Ward et al., 2001). Moreover, blocking COPII-mediated ER-exit by using a dominant-negative mutant of the COPII component Sar1 causes re-absorption of GFP-tagged Golgi enzymes to the ER, demonstrating a strong dependence of Golgi integrity on COPII-mediated ER-exit (Shima et al., 1998; Storrie et al., 1998; Ward et al., 2001). These results raised the exciting question whether the Golgi complex is an independent (static) organelle or is synthesized from ER membranes. Evidence of the later was obtained recently through monitoring the biogenesis of a Golgi complex in karyoblasts, in which the Golgi complex had been initially depleted by laser nanosurgery (Tängemo et al., 2011).

Multi-colour time-lapse imaging in S. cerevisiae also helped to shed light on the long-deliberated question of how transport within the Golgi complex is organised and the nature of its cisternae is maintained (Losey et al., 2006; Matsuura-Tokita et al., 2006). In S. cerevisiae, Golgi cisternae are not stacked as in S. cerevisiae, Golgi cisternae are not stacked as in
Box 1. Different F-techniques

F-techniques are quantitative methods that provide information of protein dynamics or protein–protein interactions and include fluorescence microscopy-based methods, such as those listed below.

FRAP

In fluorescence recovery after photobleaching (FRAP), a laser scanning confocal microscope is used to irreversibly destroy the fluorophore (e.g. GFP) that is linked to the protein of interest by photobleaching it with high laser excitation intensity (Axelrod et al., 1976). Thereafter, the region that has been photo-bleached is monitored by time-lapse microscopy to quantify the kinetics of reappearance of the fluorescent signal in the bleached area (Lippincott-Schwartz et al., 2003; Lippincott-Schwartz and Patterson, 2003).

FLIP

Fluorescence loss in photobleaching (FLIP) is related to FRAP. Area(s) within a cell are continuously photo-bleached and the kinetics of loss of fluorescence outside the bleaching area is monitored using time-lapse microscopy to determine the kinetics of exchange of fluorescent material between the bleached and non-bleached area(s). Using this approach, continuities or material exchange between organelles, such as the Golgi complex and the ER, can be revealed (Lippincott-Schwartz et al., 2003; Lippincott-Schwartz and Patterson, 2003).

iFRAP (inverse FRAP)

This method is also related to FRAP. Here, all the fluorescent labelling within a cell that is not under investigation is irreversibly destroyed by photobleaching and the kinetics of the remaining fluorescence monitored using time-lapse microscopy. In this way, for instance, transport of the labelled material can be quantified within living cells (Lippincott-Schwartz et al., 2003; Lippincott-Schwartz and Patterson, 2003).

FRET

Förster resonance energy transfer (FRET) occurs when two fluorescent molecules come in close proximity to each other (~5 nm or less), such that the donor fluorophore can transfer part of the energy of its excited state via dipole–dipole interactions to the acceptor fluorophore ( Förster, 2012). Several distinct imaging methods exist to quantify FRET in living cells, and they have been extensively used to image biochemical reactions, such as protein–protein interactions in living cells (Piston and Kremers, 2007; Zeug et al., 2012).

FCS and FCCS

Fluorescence correlation spectroscopy (FCS) and fluorescence cross-correlation spectroscopy (FCCS) are single-molecule approaches that analyse the fluctuation of fluorescence in a confocal volume that is usually generated by spot-laser illumination of the sample in a microscope. FCS has been mostly used to quantify the absolute number of molecules in a volume, as well as their diffusive and non-diffusive dynamics (Bacia et al., 2006; Chen et al., 2008). In comparison to FRAP, FCS measurements need far fewer labelled molecules and, thus, can measure molecule dynamics at concentrations that are close to physiological expression levels and with reduced exposure to light of the living cells under observation. In FCCS two types of molecule are labelled with distinct fluorophores and their fluctuations analysed within a confocal volume. If the two molecules interact with each other they will move through the volume simultaneously and generate a cross-correlation signal. FCCS has been used to monitor protein–protein interactions in living cells (Bacia et al., 2006).
et al., 2006). This simple but elegant approach revealed that each cisterna initially labelled by the cis-Golgi marker (green) changed colour to the trans-Golgi marker (red), suggesting that Golgi cisternae mature over time (Losev et al., 2006; Matsuura-Tokita et al., 2006). These results are consistent with the Golgi maturation model (Glick et al., 1997), which predicts that secretory cargo moves through the Golgi complex in cisternae that form de novo, mature from cis to trans, and then dissipate.

The examples above, which are by far not comprehensive owing to space constraints, aim to illustrate certain fundamental mechanistic aspects of ER-to-Golgi transport that would be very difficult, or even impossible, to address without the direct visualization of the factors involved inside living cells. However, despite its great potential, this approach also has limitations. First, in contrast to in vitro systems, where selected factors are combined and their interactions or functions studied, any interpretation of microscopy experiments in living cells has to deal with the complexity of the entire cell and is therefore often difficult. Second, when performing time-lapse-imaging experiments, the possibility of cellular damage or the occurrence of artefacts due to extensive exposure to fluorescence excitation light always exists and is difficult to evaluate. Third, life cell imaging requires the expression of a fluorescent marker in cells, which mostly is achieved by tagging the protein of interest with fluorescent proteins. This always bears the possibility that the tag changes the natural behaviour of the protein. In addition, the expression levels of FPs have to be controlled to ensure that cellular processes are not disrupted and FPs reflect the behaviour of their endogenous counterparts. Another problem is the difference in expression levels among individual cells, which can be partially overcome with the use of stably transfected cell lines. Physiological expression levels may be obtained by driving the expression of the cDNA under the control of the endogenous promoter of that gene and flanking regulatory regions (Poser et al., 2008).

Resembling the well-established method for GFP expression in yeast cells (Howson et al., 2005), the endogenous gene may be replaced by an FP version using the recently developed gene replacement technology in mammalian tissue culture cells (Gaj et al., 2012; Hauschild-Quintern et al., 2013; Santiago et al., 2008).

In summary, time-lapse imaging of FP in living cells has helped to reveal many unexpected aspects of the spatiotemporal organisation of ER-to-Golgi transport and the organelles involved. However, owing to its limitations in resolving structures below the diffraction of light, time-lapse fluorescence microscopy is not able to provide information on the nature of, for example, intra-Golgi transport, or the behaviour of COPI or COPII transport vesicles at donor membranes or fusion with target membranes. Similarly, molecular interactions of key regulators and structural proteins operating in ER-to-Golgi transport cannot be imaged by time-lapse fluorescence microscopy because of limitations in resolution.

**Imaging methods for advanced analyses of ER-to-Golgi transport**

To overcome the limitations of time-lapse fluorescence microscopy several more advanced microscopy techniques have been either re-discovered or newly developed in the past years.

**F-techniques**
The so-called F-techniques (see Box 1) are able to characterise quantitatively the diffusion and binding kinetics of fluorescent proteins, as well as their interactions. Although, all F-techniques, in principle, have a great potential to study the early secretory pathway, mainly fluorescence recovery after photobleaching (FRAP) and Förster resonance energy transfer (FRET) measurements have been used to explore ER-to-Golgi transport.

FRAP has been developed initially to quantitatively determine, using light microscopy, molecular dynamics such as diffusion in membranes (Axelrod et al., 1976). Since the availability of fluorescent proteins, this technology has been extensively used to study the dynamics of GFP-tagged proteins in a semi-quantitative manner and provided numerous new insights into the dynamic organisation of the early secretory pathway (Lippincott-Schwartz et al., 2003). For example, FRAP analyses of a temperature-sensitive mutant of the vesicular stomatitis virus glycoprotein (VSVG), which is widely used as a marker for secretory cargo, showed that the mobility of folded and unfolded protein within the ER is similar and highly dynamic. These results suggested that other mechanisms than protein immobilisation, for instance interactions with chaperons, must be responsible for retention of the unfolded protein in the ER (Nehls et al., 2000). More advanced approaches that combine FRAP measurements and mathematical models quantitatively describe the underlying biochemical reactions responsible for the measured recovery kinetics. This allows, for example, to determine the on- and off-rates with which cytoplasmic proteins interact with organelles in living cells (Sprague and McNally, 2005). Such modelling approaches have also been used to obtain further insights into the role of secretory cargo in the kinetics of the assembly of COPI vesicular coat proteins at ERES (Forster et al., 2006) or COPI vesicular coat proteins at Golgi membranes (Presley et al., 2002).

FRET is a non-radiative transfer of energy from an excited donor fluorophore to an acceptor fluorophore in its proximity (Fernandez and Berlin, 1976; Piston and Kremers, 2007; Zeug et al., 2012) (see Box 1). As FRET signals strongly depend on the distance between donor and acceptor fluorophore, this technique has great potential to study the spatial organisation of protein-protein interactions in ER-to-Golgi trafficking in living cells. However, to our knowledge, the number of successful applications of FRET measurements to address questions related to ER-to-Golgi transport has been rather limited. FRET measurements and confocal imaging demonstrated for the first time in living cells that oligomerisation of G-protein-coupled receptors occurs in the ER and in the Golgi, but the functional significance of this process is still not clear (Herrick-Davis et al., 2006). FRET measurements of fluorescently labelled KDEL (Lys-Asp-Glu-Leu) receptor, the retrograde transport cargo cholera toxin and COPI proteins could provide evidence for a coupling of retrograde cargo-induced oligomerisation of the KDEL receptor and its subsequent sorting into COPI vesicles (Majoul et al., 2001). FRET measurements were also used to investigate the kinetics of COPII coat complex assembly and disassembly on artificial liposomes. This revealed that the COPII pre-budding complex Sec23–Sec24 (see Table 1 for COPII components) stays bound to cargo during multiple Sar1 GTPase cycles, suggesting a model for the maintenance of kinetically stable pre-budding complex that regulate cargo sorting into COPII vesicles (Sato and Nakano, 2004).
limited by light diffraction, and structures that are smaller than ~250 nm in the xy-direction and 500 nm in the z-direction cannot be resolved. To overcome this restriction and to obtain information at the ultrastructural level, EM is the method of choice. Unfortunately, it requires fixed cells and thus temporal information on dynamic processes is difficult to obtain. In specialised experimental set-ups, where transport can be synchronised, e.g. by a shift in temperature or the addition of chemical compounds, fixation of cells at different time-points after the induction of transport can help to obtain temporal information (Bonfanti et al., 1998; Marsh et al., 2004; Trucco et al., 2004).

Combining such synchronised transport systems with electron tomography, which allows volume imaging in EM and thus also analyses of extended structures, showed that secretory cargo induces the formation of tubules between Golgi cisternae and that such tubular connections depend on the amount of cargo to be transported (Marsh et al., 2004; San Pietro et al., 2009; Trucco et al., 2004). Although these studies clearly demonstrated the existence of such tubular connections in response to cargo waves, it is not yet clear to which extent these Golgi tubules contribute to intra-Golgi transport.

Another possible way to overcome the limitation of EM in acquiring temporal information is to combine light and electron microscopy to correlative light and electron microscopy (CLEM), which first allows the visualisation of dynamic processes in living cells, followed by ultra-structural analyses in the very same fixed cells by EM (Mironov et al., 2000). One challenge when using CLEM is to identify the very same cell or structure that was observed by light microscopy for the subsequent imaging by EM. Several variations of CLEM addressing this technical challenge have been developed and used to investigate the early secretory pathway. One approach uses photobleaching with the light microscope to convert the fluorescent signal of interest into an electron-dense diaminobenzidine (DAB) precipitate that can subsequently be visualised using the electron microscope (Grabenbauer et al., 2005). This method was applied to visualise the Golgi and the ER (Gaietta et al., 2006; Meisslitzer-Ruppitsch et al., 2009), and to identify intra-Golgi COPI vesicles (Grabenbauer et al., 2005).

The structure and dynamics of formation of ER-to-Golgi carriers have also been studied using CLEM combined with tomography (Mironov et al., 2003). This study proposed that larger saccular ER-to-Golgi transport carriers form in the vicinity of COPII-coated ERES in a COPII-dependent manner without the need of budding COPII-coated vesicles. This result has added a new perspective on how ER exit and subsequent transport in the early secretory pathway might take place, and raised the question to which extent such carriers, compared with COPII-coated vesicles, mediate ER-to-Golgi transport. Immuno-electron tomography analyses have, however, shown that free COPII-coated vesicles and larger COPII-coated dumb-bell-shaped tubules of 150–200 nm in size form at ER-exit sites (Zeuschnier et al., 2006). Further work, including rigorous quantification of results, will be necessary to determine to which extent each of these carriers observed during ER exit contribute to ER-to-Golgi transport.

A further variation of CLEM was recently introduced to obtain ultrastructural information regarding the dynamics of clathrin-coated vesicle budding at the plasma membrane (Kukulski et al., 2012). First, time-lapse fluorescence microscopy was used to characterise the temporal association of fluorescently tagged regulatory factors with newly forming endocytic vesicles (Kaksonen et al., 2003; Kaksonen et al., 2005). This information was then used to correlate the ultrastructure of endocytic intermediates with protein dynamics of the regulatory factors (Kukulski et al., 2012) (see Fig. 3A). Applying a similar approach to study the early secretory pathway might help to clarify important outstanding questions, such as the role of regulators on the efficiency of the formation of COPII vesicles and the homotypic fusion of these vesicles to form VTCs (see Fig. 1), or the precise mechanisms of delivery of cargo at and through the Golgi complex.

### Super-resolution microscopy

New exciting light microscopy methods with a resolution below the diffraction limit of conventional microscopes have been developed in the past few years and were termed super-resolution microscopy (see Box 2). These methods allow to image cells in their native environment with a resolution that approaches the one obtained with EM. Different approaches to resolve transport carriers and structures that are relevant for ER-to-Golgi transport exist. For example, in stimulated emission depletion (STED), the excitation light to resolve structural details is spatially confined (Hell and Wichmann, 1994) (see Box 2), which works well in living cells and provides temporal resolutions that should allow the imaging of COPII-coated ER-to-Golgi transport carriers with a typical size between 60 and 80 nm. Accordingly, synaptic vesicles and the ER have been imaged using STED (Hein et al., 2008; Jones et al., 2011; Westphal et al., 2008; Willig et al., 2006). However, thus far, STED has not provided any new conceptual insights to ER-to-Golgi or intra-Golgi transport. One possible reason for this might be that commercial STED systems that are available to a broad community, although very powerful, do not provide sufficient resolution in the z-direction and thus do not allow to address questions regarding intra-Golgi transport mechanisms, vesicle formation at the ER or formation of vesicular tubular clusters (VTCs). Also, it should be noted that, in contrast to EM, STED and other super-resolution microscopy methods, do not provide information on the ultrastructural cellular context of the molecules analysed. In order to achieve this, multi-labelling of the samples to highlight cellular landmarks next to the structures of interest would be necessary; and this is still a challenge in super-resolution microscopy.

New approaches using structured illumination super-resolution microscopy (SIM) (see Box 2) have been developed in the past few years and come closer to fulfill the necessary requirements in

### Table 1. Minimal COPII and COPI components and their respective roles

<table>
<thead>
<tr>
<th>COPI</th>
<th>COPII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arf 1</td>
<td>Sar1</td>
</tr>
<tr>
<td>α COP</td>
<td>Sec23</td>
</tr>
<tr>
<td>β COP</td>
<td>Sec24</td>
</tr>
<tr>
<td>γ COP</td>
<td>Sec13 and Sec31</td>
</tr>
</tbody>
</table>

- **COPI**
  - Arf 1: GTPase
  - α COP: Outer or cage-like subcomplex
  - β COP: Outer or cage-like subcomplex
  - γ COP: Inner or adaptor subcomplex
  - δ COP: Inner or adaptor subcomplex
  - ζ COP: Inner or adaptor subcomplex
- **COPII**
  - Sar1: GT-Pase
  - Sec23: Inner coat, GAP activity
  - Sec24: Inner coat, cargo selection
  - Sec13 and Sec31: Outer coat, membrane bending, increases GAP activity
Fluorescence-microscopy-based imaging methods that overcome the diffraction limit of light microscopes (~250 nm in xy direction and ~500 nm in z direction) are referred to as super-resolution microscopy techniques. They include structured illumination microscopy (SIM), stimulated emission depletion (STED) microscopy and localisation microscopy methods, such as photoactivated localisation microscopy (PALM), stochastic optical reconstruction microscopy (STORM) or ground-state depletion microscopy followed by individual molecule return (GSDIM). Several other comparable methods have been developed, but are not discussed here owing to space constraints.

**SIM**

In SIM, a series of images with a stripe illumination at different, well-defined positions are recorded. Analysis of the signal variations between the different images allows to computationally resolve structures with about twice the resolution in all axes compared to conventional microscopy (Gustafsson, 2000; Gustafsson, 2008). The method is well suited for life cell imaging with all of the fluorescent labels currently available.

**STED**

In STED microscopy (Hell and Wichmann, 1994), a diffraction-limited excitation beam is superimposed by a donut-shaped STED beam that, in comparison to the excitation beam, is red-shifted and quenches excited molecules by stimulated emission depletion in the periphery of the excitation focal spot. This way, resolutions as low as 30 nm and the possibility of life cell imaging have been reported (Westphal et al., 2008).

**Localisation super-resolution microscopy**

Localisation microscopy methods (Patterson et al., 2010) take advantage of the possibility to localise single fluorescent molecules at very high precision, down to the nanometer scale. With these methods, fluorescent molecules, such as photo-activatable or photo-switchable fluorescent proteins, are randomly switched on and imaged as single molecules by using camera-based microscopy systems before they are switched off again. Repeating this cycle up to several thousand times allows the construction of super-resolution images of the fluorophore distribution. Examples of current localisation techniques that function on the basis of switching the observed fluorescent dyes between their dark and bright states are, for instance, PALM (Betzig et al., 2006; Hess et al., 2006), STORM (Rust et al., 2006) or GSDIM (Fölling et al., 2008).

Developments showed that it is possible to achieve two-colour 3D imaging of clathrin-coated vesicles with a spatial resolution of ~120 nm (Fiolka et al., 2012). Although these exciting recent developments indicate that SIM is a promising technique with which to obtain multi-label life cell imaging using common fluorophores, the spatial and temporal resolution of SIM systems that are currently available to a larger community (~100–150 nm) is not yet sufficient to allow life cell imaging of small carriers, such as COPII- or COPI-coated vesicles with sufficient temporal resolution that is necessary to track them during ER-to-Golgi transport.

Another super-resolution approach uses stochastic single-molecule localisation (here referred to as localisation microscopy; see Box 1) (Lidke and Lidke, 2012; Patterson et al., 2010; Sengupta et al., 2012; Toomre and Bewersdorf, 2010). Photo-switchable or photo-activatable probes are used to generate
thousands of images, in which single molecules can be localised with very high precision. These images are then used to reconstruct the density labelling of the fluorescent marker of interest in the cell with a resolution down to 10 nm (Patterson et al., 2010). A major drawback of super-resolution localisation microscopy is that imaging of fast events in the (sub)-second range, such as those important in ER-to-Golgi transport, is difficult because the number of single-molecule events that can be imaged within this time-frame is often not sufficient to allow a faithful reconstruction of the distribution of the molecule of interest in living cells. However, imaging of slowly moving structures, such as adhesion complexes (Shroff et al., 2008), mitochondria, lysosomes or the ER (Shim et al., 2012), by using stochastic super-resolution methods has been demonstrated. Performance comparison of total internal reflection fluorescence (TIRF) microscopy and the localisation microscopy approach ground-state depletion microscopy followed by individual molecule return (GSDIM) (see Box 2) in imaging COPII and COPI structures at ERES is shown in Fig. 3B. Similar to STED, initial attempts of using localisation microscopy had their limits in that resolution in z-direction did not match the one achieved in xy-direction. Recent developments, however, have overcome these limitations and demonstrated studies localisation microscopy applications with a significantly improved resolution in z-direction (Huang et al., 2008; Shtengel et al., 2009; Xu et al., 2012). Studies investigating the early secretory pathway might benefit greatly from super-resolution imaging as this technique may help to answer important outstanding questions, such as what underlies formation, fission and fusion of transport vesicles or carriers that have a role in the early secretory pathway, how are organelles such as the Golgi complex organised, and what are the links between transport vesicles and the microtubule cytoskeleton. However, to address these questions, it will be necessary to achieve the appropriate spatial and temporal resolution in life cell super-resolution imaging. As some transports carriers involved in ER-to-Golgi transport are only between 60 and 80 nm, and need to be tracked at a sub-second resolution, life cell super-resolution imaging remains a challenge to study ER-to-Golgi transport.

**Imaging ER-to-Golgi transport at the systems level**

As mentioned above, imaging ER-to-Golgi transport by using different microscopy techniques in living cells has revealed its dynamic nature and led to the proposal of new concepts with regard to the organization of the early secretory pathway. However, the imaging approaches used to reveal these new concepts, such as time-lapse imaging or FRAP analyses, have only contributed to a limited extent to the identification or characterization of factors that regulate the early secretory pathway at the systems level. To understand the early secretory pathway at the systems level, including its regulation by responses to e.g. extracellular stimuli or differentiation processes, it is necessary to first identify the factors involved in a comprehensive manner. Once this is achieved, it is important to quantitatively determine how and when these factors interact with each other in order to formulate a quantitative model that describes the regulation of the early secretory pathway.

In a first step towards this goal, proteomic approaches were used to comprehensively identify secreted proteins as well as proteins of the organelles that encompass the secretory pathway (Gannon et al., 2011; Gilchrist et al., 2006; Meissner et al., 2013). However, these approaches require biochemical purification of the organelles, which removes them from their cellular context and, therefore, the proteins identified in such studies may not represent all the proteins that associate with the secretory pathway and related organelles in unperturbed intact cells.

Systematic localisation and unbiased functional characterisation of uncharacterised GFP-tagged human cDNAs in living cells have been used as an alternative to identify potential new regulators of the early secretory pathway (Simpson et al., 2000; Starkuviene et al., 2004; Starkuviene and Pepperkok, 2007). The involvement of a gene in the early secretory pathway can also be investigated in intact cells, by knocking it down using RNA interference (RNAi) and analysing the effect on trafficking and/or the morphology of the secretory pathway components. A number of such RNAi-based screens have been performed in metazoans (Bard et al., 2006; D’Ambrosio and Vale, 2010; Farhan et al., 2010; Kondylis et al., 2011; Simpson et al., 2012; Wendler et al., 2010). For example, two genome-wide screens identified new genes with a role in protein secretion by first analysing the effects of gene knockdown on protein secretion by using enzymatic bulk readout analysis, followed by systematic microscopy-based analyses to better understand the role of the identified genes in regulating the early secretory pathway or Golgi integrity (Bard et al., 2006; Wendler et al., 2010).

To achieve quantitative systematic analyses in high-throughput microscopy, all steps of the experiment, including sample preparation, image acquisition and single-cell quantitative analyses need to be automated and integrated (Pepperkok and Ellenberg, 2006). Such an automated, integrated approach has recently been used to identify, at genome level, those genes that, when knocked down, cause alterations in biosynthetic protein trafficking (Simpson et al., 2012) and organelle integrity (Farhan et al., 2010; Kondylis et al., 2011; Simpson et al., 2012). Farhan and colleagues investigated the role of human kinases and phosphatases in the distribution of membrane markers of the early secretory pathway, and showed that growth factor signalling regulates ER export through ERK2. Besides the MAPK signalling pathway, a number of other signalling networks, such as those involving insulin, phosphatidylinositol or toll-like receptor signalling, were also found to have a role in the regulation of the early secretory pathway (Farhan et al., 2010).

Using a genome-wide RNAi screen, Simpson and colleagues showed that several GTPases and GTPase-binding proteins have a role in the regulation of ER-to-Golgi transport and organelle integrity (Simpson et al., 2012). Moreover, their work showed that EGF-mediated signalling from the cell surface regulates the efficiency of ER-exit, as demonstrated by knockdown of the components of a network consisting of EGF signalling factors and the EGF receptor (Simpson et al., 2012). Similarly, a recent RNAi screen in *Drosophila* S2 cells that targeted over 100 genes that had been selected using bioinformatics has shown a potential link between the early secretory pathway and cell metabolism (Kondylis et al., 2011).

Together, these systematic analyses using high-throughput microscopy suggest that the regulation of the secretory pathway is tightly coupled to other cellular processes at multiple levels and that only an *in vivo* approach will be able to unveil these networks and regulatory processes. The genes that, in these various RNAi screens, were shown to be involved in the regulation of the early secretory pathway represent an excellent basis for follow-up studies to further investigate their role and mechanism of action. The first step to achieve this might be high-throughput microscopy in living cells at large scale, to evaluate the effect of gene
knockdown on the dynamics of transport and organelle alteration at the boundary between ER and Golgi, similarly to what has been demonstrated for the endocytic pathway and cell division (Collinet et al., 2010; Neumann et al., 2010).

**Future perspectives**

Sequencing of the complete human genome gave biologists a better understanding of cellular processes and their regulation at a systems level. The option to express fluorescent proteins and fluorescent probes allows to visualise almost any cellular structure by using fluorescence microscopy in living cells and to access proteins in their natural environment, thereby providing temporal and spatial information of their function (Pepperkok and Ellenberg, 2006). Although these approaches, together with correlative light and electron microscopy analyses have, indeed, changed our view on the dynamics and molecular organisation of the early secretory pathway, many future challenges and opportunities remain. One of them will be to develop robust tools to image lipids in living cells. Owing to the lack of such tools in the past, our knowledge of the role of lipids in the early secretory pathway is fairly limited. Which lipids at what concentration, facilitate the formation of tubular or vesicular carriers or participate in regulating ER-to-Golgi transport remain open questions.

One possible way to image the distribution and dynamics of lipids indirectly is the use of fluorescently labelled proteins with a lipid-binding domain that specifically recognises a particular class of lipids, such as inositides, diacylglycerol or phosphatidylserine (Sarantis and Grinstein, 2012; Schultz, 2010). Using this approach, in combination with biochemical analysis, has revealed that PtdIns(4)P is required for Golgi integrity and protein sorting (Pao and Mayinger, 2012) and that it might also have a role in the formation of ERES (Blumental-Perry et al., 2006). To improve this indirect approach to image lipids under non-physiological conditions, it will be necessary to use living cells and directly label lipids under conditions as similar as possible to physiological conditions (Schultz, 2010). Strategies of chemical biology, which involve organic synthesis of the lipid of interest, have been developed (Mentel et al., 2011; Schultz et al., 2010) and demonstrated recently that PtdIns(3)P induces fusion of early endosomes (Subramanian et al., 2010).

Combining life cell imaging techniques with other methods, such as RNAi, small-molecule-based perturbation, overexpression analysis or subcell manipulation such as laser nanosurgery, can result in an invaluable amount of quantitative information. Recent developments of super-resolution microscopy to cell life imaging, 3D and improved speed of data acquisition (Dedecker et al., 2012; Eggelin et al., 2013; Gao et al., 2012; Hein et al., 2008; Lidke and Lidke, 2012; Planchnon et al., 2011; Shim et al., 2012; York et al., 2011), might make it possible to image the molecular organisation of the early secretory pathway in living cells at so-far unmatched resolution. Moreover, the combination of super-resolution microscopy with other methods, such as FRET (Cho et al., 2013) or correlative life cell imaging (Bálint et al., 2013), might reveal details of intra-Golgi transport or the formation of transport carriers for large cargo at ERES that, to date, cannot be accessed owing to the resolution limits of conventional fluorescence time-lapse microscopy. Until now, quantitative high-throughput imaging in living cells at the genome scale has only been achieved in a few cases (Collinet et al., 2010; Neumann et al., 2010) and remains technically challenging. However, availability of the technology and the success of these studies in revealing quantitative descriptions of phenotypes on the basis of organelle and molecule dynamics in a comprehensive manner will certainly stimulate its application to other biological questions, such as the organisation and regulation of the early secretory pathway.

Extending high-throughput imaging to include FRAP, FRET or super-resolution microscopy analyses of the early secretory pathway is still very challenging; however, it has great potential in providing comprehensive and quantitative understanding of how the early secretory pathway is regulated. The foundations to achieve this goal have already been laid with the recent development that allows feedback-control of microscopes (Conrad et al., 2011; Tsukada and Hashimoto, 2013), as well as with automatic high-throughput FRAP analyses on specific cellular structures as that are automatically recognised by online image analysis (Conrad et al., 2011). With such systematic quantitative data sets at hands, we anticipate that it is possible to draft the first quantitative models of the protein networks that underlie the molecular organisation of the early secretory pathway.

**Acknowledgements**

We thank Wanda Kukulski, Marko Kaksonen and John Briggs (EMBL, Heidelberg, Germany) for providing Fig. 3A, and Stefan Terjung (EMBL, Heidelberg, Germany) for acquiring the data for Fig. 3B. We apologise to all colleagues whose work was not cited due to space restrictions.

**Funding**

F.V. and R.P. are supported by an EU Systems Microscopy network of excellence grant FP7/2007-2013-258068.

Supplementary material available online at http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.121061/-/DC1

**References**


A toolbox for assessing protein location and function.


Giepmans, B. N., Adams, S. R., Ellisman, M. H. and Tsien, R. Y.


A modular design for the clathrin- and actin-mediated endocytosis machinery.

Cell 123, 305-320.


PLoS ONE 6, e17173.


Cell 150, 588-598.


J. Cell Sci. 125, 2571-2580.


Trends Cell Biol. 16, 61-64.


Science 300, 87-91.


Nature 441, 1002-1006.


EMBO J. 1, 139-151.


Nat. Methods 5, 322-333.


J. Microsc. 232, 322-333.


Cell. Mol. Life Sci. 70, 131-145.


Cell 25, 165-176.


Cell 25, 165-176.


**Movie 1. Time lapse imaging of ERES.** COP II protein Sec31, fluorescently tagged with YFP (Sec31-YFP) and stably transfected in Hela cells, was imaged using a spinning disk confocal microscope; 3D stacks covering 59 different image planes were acquired every 15 minutes. Note how the ERES reduce significantly in number and intensity during mitosis and reappear at later stages of cell division. Scale bar: 10 µm. See text and figure 2A for more details.

**Movie 2. Life cell imaging of ERES and secretory cargo.** The Secretory cargo VSVG (vesicular stomatitis virus glycoprotein) labeled with CFP (CFP-VSVG) was co-imaged with the COP II component Sec24D labelled with YFP (YFP-Sec24D). Initially the secretory cargo (green) co-localises to ERES (red); subsequently it segregates and moves towards the Golgi complex without any apparent labelling of Sec24D. Scale bar: 2.5 µm. The original movie was published in Stephens et al., 2000.