Measuring the effects of α_1-antitrypsin polymerisation on the structure and biophysical properties of the endoplasmic reticulum

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Word count excluding legends and references: 2268

Running title: α_1-antitrypsin effects on the ER

Key words: α_1-antitrypsin, ER overload, ER stress, ROVI, microviscosity

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Abstract

An important function of the endoplasmic reticulum (ER) is to serve as a site of secretory protein folding. When the accumulation of misfolded proteins threatens to disturb luminal homeostasis, the cell is said to experience ER stress. By contrast, the accumulation of well-folded proteins inside the ER leads to a distinct form of strain called ER overload. The serpins comprise a large family of proteins whose folding has been studied in great detail. Some mutant serpins misfold to cause ER stress, while others fold but then polymerise to cause ER overload. We discuss recent advances in the use of dynamic fluorescence imaging to study these phenomena. We also discuss a new technique that we recently published, rotor-based organelle viscosity imaging (ROVI), which promises to shed more light on the biophysical features of ER stress and ER overload.

Introduction

Dysfunction of the endoplasmic reticulum (ER) is pathogenic in many diseases including cancer (Clarke et al., 2014; Dalton et al., 2013), diabetes (Thomas et al., 2010), cirrhosis (Chambers and Marciniak, 2014) and dementia (Roussel et al., 2013a). In recent years, ER dysfunction has also been implicated in the pathogenesis of α_1-antitrypsin deficiency (Davies et al., 2009; Greene et al., 2016; Kroeger et al., 2009; Lawless et al., 2004). Newly synthesised secretory proteins are folded and modified within the ER, but these maturation processes require carefully orchestrated biochemical and biophysical changes to the client secretory proteins (Roussel et al., 2011).

The crowded molecular environment of the ER therefore requires a dedicated machinery of ER-resident molecular chaperones, folding factors and an ER-associated protein degradation machinery to ensure that only correctly folded proteins are secreted (Marciniak and Ron, 2006). If these processes fail, for example when the truncated, terminally misfolded Null Hong Kong mutant of α_1-antitrypsin accumulates, the cell experiences a toxic state termed ER stress (Walter and Ron, 2011). The cell is defended against ER stress by the activation of a homeostatic pathway termed the “unfolded protein response” (UPR) (Dalton et al., 2012; Marciniak and Ron, 2006; Walter and Ron, 2011). In contrast, when well-folded proteins accumulate within the ER, a less well-understood ER overload response (EOR) is activated (Davies et al., 2009; Roussel et al., 2013b) with pro-inflammatory effects mediated by the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Ekeowa et al., 2011). We showed that activation of NF-κB in this setting requires the release of ER calcium (Davies et al., 2009). We also showed that mutants of many serine protease inhibitors (serpins) that result in protein polymerisation, e.g. Z-α_1-antitrypsin (E342K) or neuroserpin (S52R and G392E), trigger this ER overload pathway when their polymers accumulate within the ER (Davies et al., 2009; Ekeowa et al., 2009; Kroeger et al., 2009).

While ER stress can impair protein secretion and, if unresolved, result in cell death, comparatively little is known about the pathogenesis of ER overload. The serpin superfamily of proteins provides many excellent models for the study of ER dysfunction because well-characterized variants exist for several members (e.g. α_1-antitrypsin and neuroserpin) that cause either ER stress or ER overload depending upon the nature of the mutation (Belorgey et al., 2011; Ekeowa et al., 2009; Irving et al., 2011; Roussel et al., 2011). We have shown that polymers of Z-α_1-antitrypsin accumulate in electron-dense vesiculated inclusions composed of ER with markedly distorted architecture (Fig 1)(Dickens et al., 2016; Ordonez et al., 2013). Despite these changes to ER architecture, ER stress does not immediately occur (Dalton et al., 2013; Davies et al., 2009; Ferlay et al., 2010; Kroeger et al., 2009); instead, ER overload sensitizes cells to exaggerated ER stress if the cells should experience a second insult (Davies et al., 2009; Graham et al., 1990; Kroeger et al., 2009; Lawless et al., 2004; Ordonez et al., 2013).

ER inclusions

Previous studies using subcellular fractionation suggested that ER inclusion bodies are physically separated from the main ER network, but the mechanism for this has remained unclear (Graham et al., 1990). The formation of inclusions is favoured by circumstances of impaired protein degradation. For example, cells deficient in authorphagy owing to deletion of the *Atg5* gene, showed accumulation of Z-α_1-antitrypsin-containing inclusions (Kamimoto et al., 2006). Similar results were obtained during inhibition of the proteasome ((Kroeger et al., 2009) and unpublished data). It has been suggested that the formation of inclusions in cells expressing Z-α_1-antitrypsin is a protective mechanism necessary to maintain ER functionality (Granell et al., 2008). While inclusions where observed to contain many ER proteins, it was reported that they lacked the transmembrane lectin chaperone calnexin (Granell et al., 2008). This was taken to indicate that inclusions are functionally different to tubular ER. However, the use of fluorescently tagged calnexin fusion proteins has not

supported this contention, at least in heterologous expression systems such as Chinese Hamster Overy (CHO) cells, in which calnexin-mCherry highlights the membranes of inclusionss (Fig 1A) (Dickens et al., 2016). Instead, it appears that inclusions bare strong functional similarity to 'normal' ER apart from having a distorted morphology. Further studies were therefore necessary to understand how ER overload leads to increased sensitivity to ER stress.

Using photobleaching techniques – fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) – we demonstrated that accumulation of serpin polymers within the ER impairs the mobility of other soluble marker proteins, including the major ER chaperone BiP (Dickens et al., 2016; Ordonez et al., 2013). This correlates with increased susceptibility of affected cells to ER stress caused by an independent second insult. Based on these observations, we hypothesized that impaired mobility of chaperones during ER overload is responsible for this hypersensitivity to ER stress.

Protein mobility – fragmented ER

To explore this hypothesis further, we generated fluorescently tagged control and polymerogenic serpins, and fluorescently tagged chaperones. YFP-tagged Z-α_1-antitrypsin formed intracellular inclusions indistinguishable from those seen with untagged protein (Fig 1A)(Dickens et al., 2016). Because the 2C1 antibody, which detects clinically relevant polymers of antitrypsin *in vivo*, recognized these inclusions we concluded that tagged protein still faithfully recapitulates the disease mechanism. In this model, photobleaching of YFP-Z-α_1-antitrypsin within a single inclusion has little immediate effect on adjacent inclusions and photobleached regions remain visible for minutes (Fig 1B upper panels)(Dickens et al., 2016). But when an inert ER fluorescent marker protein, ER-mCherry, was expressed with polymerised Z-α_1-antitrypsin, the soluble ER-mCherry appeared to move freely within and between inclusions (Fig 1B lower panels). This

suggests that polymerized YFP-Z-α_1-antitrypsin forms a hydrogel through which other proteins can diffuse. When we used a fluorescently tagged ER chaperone BiP-mCherry (Ostrovsky et al., 2009) or a mutant defective in substrate-binding BiP-V461F-mCherry (Chambers et al., 2012), we saw that the functional chaperone could also diffuse through the hydrogel of Z-α_1-antitrypsin polymers, albeit with a slower mobility than ER-mCherry. Surprisingly, although the polymers of Z-α_1-antitrypsin are thought to be well-folded, at least some of this reduced mobility reflects BiP's capacity to bind substrate, since the BiP-V461F mutant moved more rapidly than the functional chaperone (Dickens et al., 2016).

Subcellular fractionation suggested that ER inclusion bodies are physically separated from the main ER network, but whether this was true within intact cells remained unproven for many years (Granell et al., 2008). To address this, we performed FLIP experiments in which the ER membrane was marked with a fluorescent protein. Following repeated photo-bleaching of part of one inclusion's membrane, marked differences in the rate of fluorescence bleaching were observed between patches of membrane in the same inclusion or another equally distant inclusion (Fig 1C)(Dickens et al., 2016). This suggested that in live cells, inclusions are physically separated from one another. More recently, similar results were replicated in hepatocytes derived from human induced pluripotent stem cells, which spontaneously develop similar fragmentation of the ER when Z-α_1-antitrypsin is expressed under the control of its endogenous promoter (Segeritz et al., 2018). When we performed 3-dimensional electron micrography on cells heterologously expressing Z-α_1-antitrypsin, we saw no evidence of tubular connections between inclusions even at a resolution of 18 nm (Fig 1D). It appears that inclusions form as the ER vesiculates during the accumulation of polymers of Z-α_1-antitrypsin. The mechanism for this is not fully understood, but it is noteworthy that proteins including the reticulons and atlastins which maintain the tubular ER network, are excluded from inclusions (Fig 1E)(Dickens et al., 2016). Since these ER structural proteins bind only to membranes of high curvature it is

tempting to speculate that flattening the ER's curves by depositing increasing amounts of Z-α_1-antitrypsin polymer may make the membrane unfavourable to their binding (Fig 1F).

The exchange of material between inclusions is dependent upon cytosolic factors. Washout of cytosolic proteins by treating cells with digitonin, which at titrated concentrations selectively punctures the plasma membrane, led to the inhibition of exchange between inclusions of fluorescently tagged proteins ((Dickens et al., 2016) and Figure 8 therein). This could be rescued by supplementation with exogenous cytosol. Material exchange could also be blocked by the SNARE inhibitor N-ethyl maleimide or by the expression of a dominant-negative mutant of Sar1, a GTPase required for COPII vesicle formation and protein transport from the ER. In addition, the appearance of Z-α_1-antitrypsin in ERGIC53 positive structures following treatment with brefeldin A is consistent with recycling via the ER Golgi intermediate compartment (ERGIC) during exchange of material between inclusions.

Maintaining the connectivity of the ER is a costly dynamic process that requires the action of many ER shaping proteins and fusogens (Chen et al., 2013). The benefits of an extensive tubular network are likely to be manifold, but a plausible example would be to maintain mixing of luminal contents necessary for proper access of chaperones to their client proteins. If one region of the ER were to experience a relative deficit of chaperones, for example because of local variations in the rate of protein synthesis, the ER stress sensors embedded in these regions would be activated. In a cell in which the integrity of the ER network has been compromised by vesiculation, such local differences would likely be amplified. Even a single isolated inclusion, if experiencing local defects in matching chaperone function to client load, could potentially subject the whole cell to ER stress signaling. We therefore suspect that mixing of luminal contents through recirculation via the ERGIC may be a protective mechanism against ER stress since it would tend to limit heterogeneity between physically separated portions of vesiculated ER. It is now necessary for techniques to be developed that will permit the detection of ER stress signaling with subcellular resolution so these hypotheses can be tested.

Protein mobility – hydrogels

Hydrogels made of polymerized macromolecules are gaining increased attention owing to their relevance both in bioengineering and in fundamental cellular processes (Boeynaems et al., 2018; Madl et al., 2018). The lattice-like structure of hydrogels can allow the movement of soluble factors including proteins. However, the physical nature of the hydrogel has complex effects on the movement of proteins, since it can both hinder diffusion by acting as a partial barrier, but also speed the movement of large molecules through size exclusion effects. Existing techniques, such as FRAP and fluorescence anisotropy have been used to probe the effects of some physical parameters of hydrogels, such as polymer density and cross-linking, on rotational and translational diffusion of model proteins, but important questions remain (Bertz et al., 2013; Rapp et al., 2017). To what extent, for example, is protein mobility affected by changes to ER microviscosity rather than through effects on protein binding or crowding?

Microviscosity

Such considerations are important because the efficiency of protein folding and the fidelity of protein-protein interactions that drive chaperone-assisted protein folding are dependent on diffusion at the nanoscopic scale. *In vitro*, these processes have been shown to depend strongly on biophysical characteristics such as macromolecular crowding and microviscosity. Microviscosity governs both the translational and rotational motion of molecules inside the cell (Kao et al., 1993). The importance of this is demonstrated by changes in plasma membrane microviscosity that alter the motility of endothelial cells in response to growth factors (Ghosh et al., 2002). The dynamics of i*n vitro* protein folding and enzyme activity are also sensitive to changes in microviscosity (Ansari et al., 1992; Dhar et al., 2010). However, whilst macromolecular crowding is a known modulator of protein folding *in vitro*, its effects are thought to be complex and reports suggest it exerts

opposing influences on different processes in the protein-folding pathway (Gershenson and Gierasch, 2011).

Until now, it has not been possible to measure the microviscosity inside a cellular organelle in an accurate and reliable manner. We therefore recently developed a technique that we called rotor-based organelle viscosity imaging (ROVI) (Fig 2)(Chambers et al., 2018). Molecular rotors are small molecules with an internal axis of rotation that respond optically to the microviscosity of their environment. When increased microviscosity hinders their internal rotation, the average time taken for a photon to be emitted following excitation is changed (Kuimova et al., 2008). This increase in so-called "fluorescence lifetime" has the advantages that it is independent of fluorophore concentration and it can be imaged using specialized microscopes. We synthesized a rotor comprising a microviscosity sensitive dye fused to a HaloTag ligand. When this was added to cell expressing Halo protein within the cytosol, ER or nucleus, the dye was selectively accumulated at those sites. The system faithfully reports local microviscosity caused by, for example, changes in cell swelling, and we are currently using it to measure the effect of Z-α_1-antitrypsin polymerization on ER microviscosity. Crucially, ROVI is insensitive to molecular crowding (Chambers et al., 2018). This new technique promises to enable studies that will elucidate the mechanistic links between biophysical properties of Z-α_1-antitrypsin polymers and their consequences for ER function.

Acknowledgements

This work was funded by the MRC, Alpha-1 Foundation, British Lung Foundation, Academy of Medical Sciences, Addenbrooke's Charitable Trust, the Cambridge BRC and Papworth Hospital.

Legends

Figure 1 The endoplasmic reticulum remains functionally connected by vesicular transport following its fragmentation in cells expressing Z-α_1-antitrypsin

(A) Integral membrane lectin chaperone calnexin (CNX)-mCherry decorates the membranes of Z-α_1-antitrypsin-containing inclusions. Bar = 5μm. **(B**) Z-α_1-antitrypsin forms a hydogel through which small ER resident proteins can diffuse. High laser intensity was used to bleach a region of interest within an inclusion of a cell expression YFP-Z-α_1-antitrypsin. YFP-Z-α_1-antitrypsin proved to be relatively immobile over a period of >2 min; while, mCherry was far more mobile, completely bleaching throughout the inclusion but recovering over 2 min. Bar = 10 μm. **(C)** Repeated high intensity laser illumination of cells expressing untagged Z-α_1-antitrypsin, led to bleaching of the ER membrane marker cytERM-msfGFP preferentially within the same inclusion. Graph illustrates cytERM-msfGFP fluorescence at bleached area (blue); two equidistant regions of interest, one on the same inclusion (red); one on an adjacent inclusion (green); and another on a distant inclusion (purple). Inset photomicrograph showing whole cell and four high-powered views of bleaching area at times i-iv marked on graph. Bars=10μm. **(D)** CHO cell expressing YFP-Z-α_1-antitrypsin was subjected to serial block-face scanning electron microscopy then 3D projection rendering was preformed. **(E)** GFP-reticulon 4a (green) and mCherry-ER (red) fail to co-localise in CHO cells untagged M or Z-α_1-antitrypsin. Bar =10μm. Reproduced from (Dickens et al., 2016). **(F)** Schematic representation of wedge-shaped reticulon protein (green) inserting into membrane of a tubule of ER (grey membrane, red lumen: upper panel), but owing to flatter morphology of expanded inclusions, the integration of the reticulon into the ER membrane is disfavoured (lower panel).

Figure 2 An Optical Technique for Mapping Microviscosity Dynamics in Cellular Organelles - ROVI

(A) Model illustrating BODIPY-HaloTag bound to HaloTag protein. **(B)** Fluorescence lifetime of BODIPY-HaloTag protein in water/glycerol solutions. **(C)** Colour coded fluorescence lifetime imaging microscopy (FLIM) of COS7 cells expressing HaloTag in the cytosol (cyto), ER, and mitochondrial matrices (mito). Cells imaged in isotonic, hypotonic, or hypertonic culture conditions. Reproduced from (Chambers et al., 2018).

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Figure 1

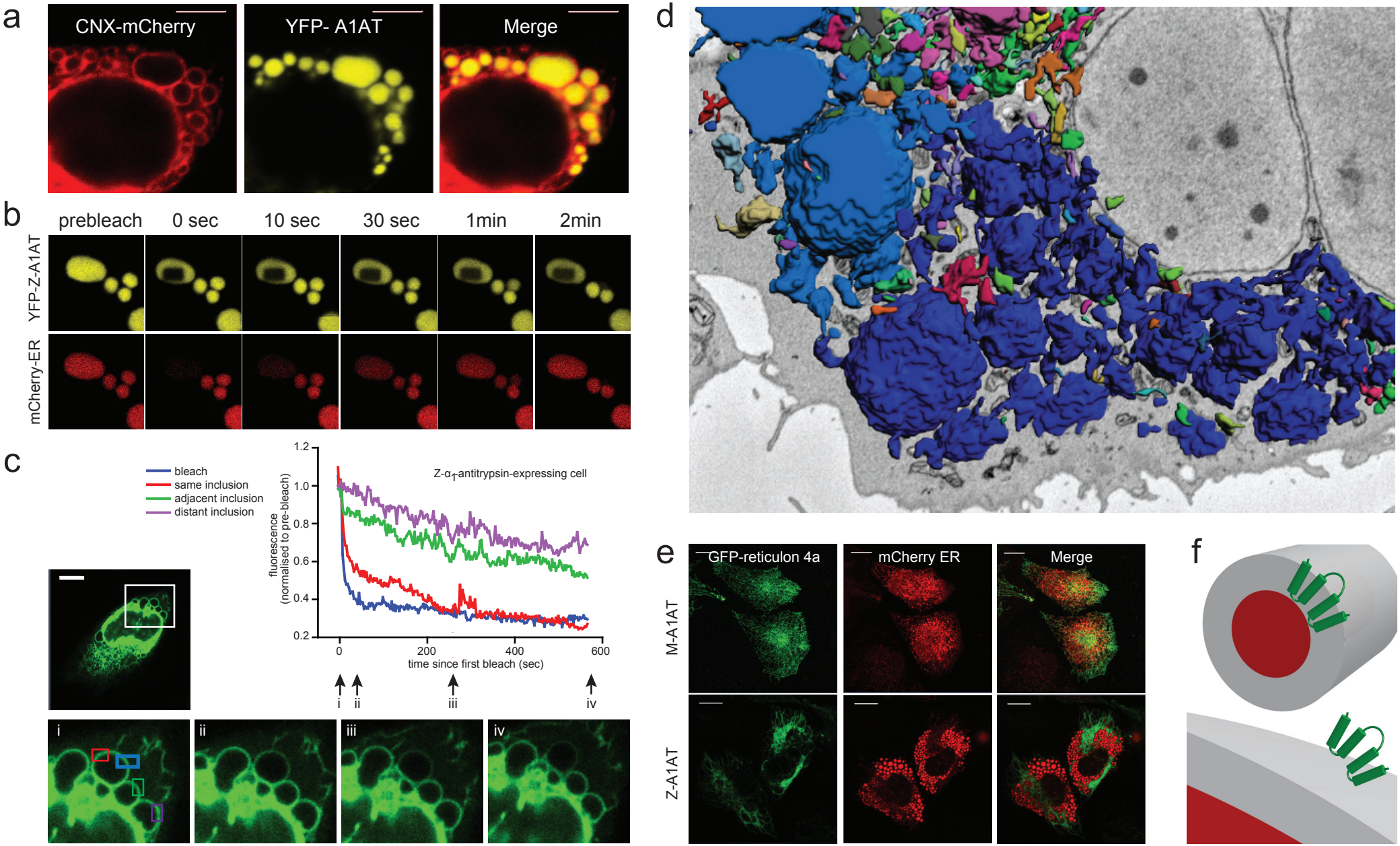
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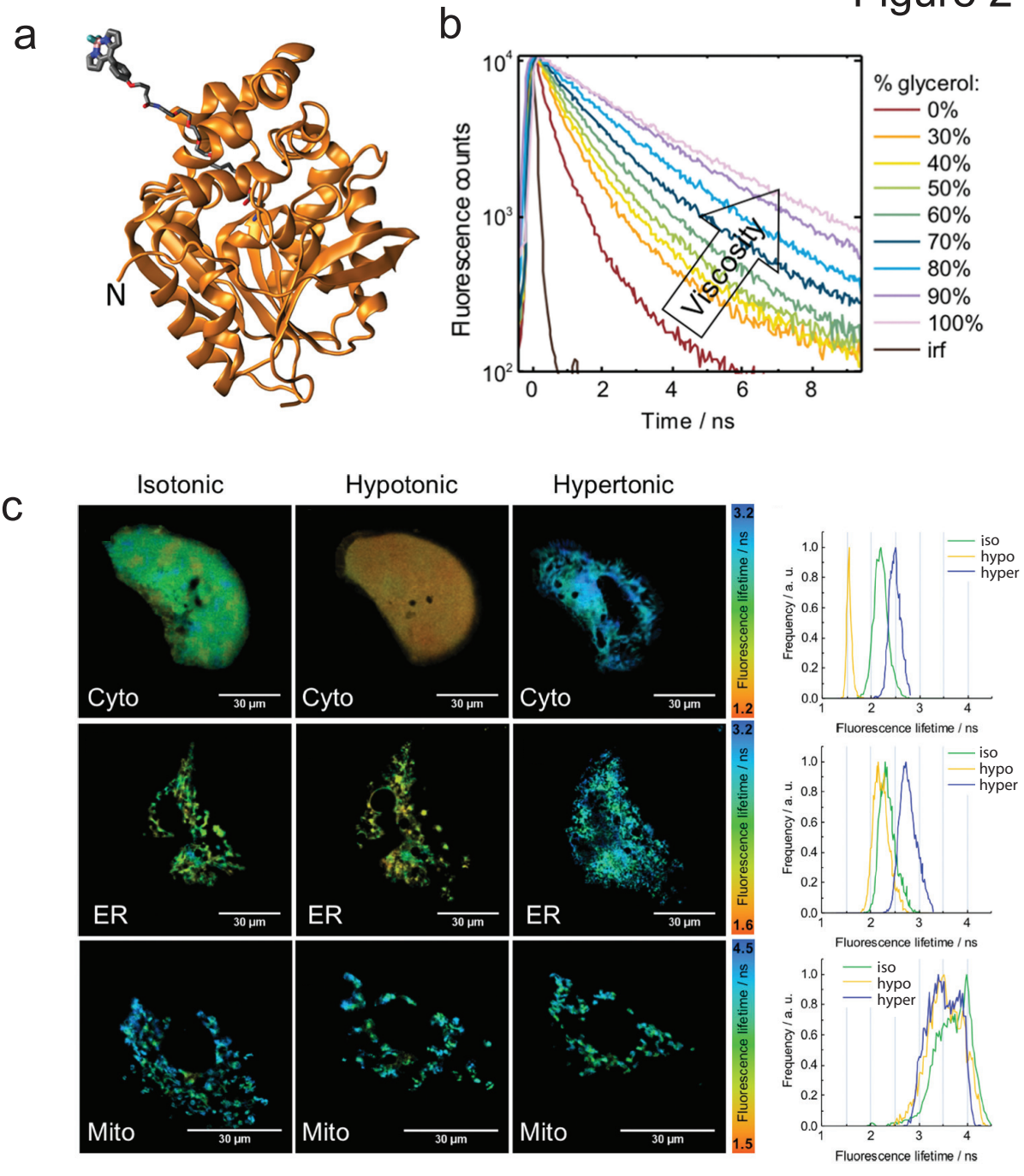
Figure 2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