Imaging nuclear, endoplasmic reticulum and plasma membrane events in real time

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Abstract

Live cell imaging can provide important information on cellular dynamics, however the full utilisation of this technology has been hampered by the limitation imaging reagents. Metal-based complexes have the potential to overcome many of the issues common to many current imaging agents. The rhenium (I) based complex *fac*-[$Re(CO)_3$(1,10-phenanthroline)(4-pyridyltetrazolate)], herein referred to as ReZolve-ER^{TM}, shows promise as a live cell imaging agent with rapid cell uptake, low cytotoxicity, resistance to photobleaching and compatibility with multicolour imaging. ReZolve-ER^{TM} localised to the nuclear membrane/endoplasmic reticulum (ER) and allowed the detection of exocytotic events at the plasma membrane. Thus, we present a new imaging agent for monitoring live cell events in real time, which is ideal for imaging either short or long time courses.

Introduction

One of the most exciting advances in cell biology has been the development of technology for live cell imaging, which enables the visualisation of molecular events in real time. However, while there have been significant advances in spatial and temporal resolution imaging, with for example spinning disk, fast scanning and super resolution microscope technologies [1,2], the field of imaging reagents has struggled to keep pace. This is partially because the imaging reagent market is undergoing a quantum shift from imaging reagents that require cell fixation, which is known to generates significant artefacts [3], to those that enable real time live cell imaging. Reagents for live cell imaging ideally should exhibit *in situ* stability, optimal emission/photophysical properties, a large Stokes shift, resistance to photobleaching, capacity for multicolour/multiple probe imaging and most importantly low toxicity [4]. Consequently, there is a very high demand for imaging reagents that meet these criteria to enable effective live cell imaging without perturbing cellular mechanisms.

There are a range of technical approaches for live cell imaging, including endogenous fluorescence, genetic expression systems, quantum dots and small fluorescent molecules.

Endogenous fluorescence has been effectively used for *in vitro* live cell and *intravital* imaging [5,6], and while this mode of imaging does not require exogenous labelling there are only a limited number of molecules capable of generating detectable endogenous fluorescence [7]. Furthermore, endogenous fluorescence (often referred to as autofluorescence) can actually be a hindrance when combined with specific microscopy imaging techniques [8]. While GFP expression systems revolutionised the field of mammalian cell biology [9,10], this technology requires genetic manipulation of the target. Moreover, the GFP molecule is large and can cause steric problems that influence molecular function [11]. In addition, many molecular targets, including carbohydrates and lipids are not amenable to this technology. While the use of quantum dot imaging is rapidly increasing, this nanocrystal technology can be prone to particle breakdown, toxicity and high production costs [12]. To date the majority of the commercially available reagents for fluorescence imaging have been based on organic compounds, like BODIPY. These compounds can suffer from a range of issues including concentration dependent fluorescent shifts, photobleaching and cytotoxicity. To address the growing need for specific, high quality imaging reagents that do not affect cell viability, researchers have been exploring luminescent metal complexes, such as those of Re(I), Ru(II), Ir(III), Pt(II) and the trivalent lanthanides [13-16]. Metal complexes have the potential to overcome a number of the pitfalls associated with organic fluorophores, as they are typically resistant to photobleaching, allowing excitation for longer periods of times with respect to organic fluorophores. Moreover, the triplet multiplicity nature of the excited states of these species implies that their excited state lifetime is usually elongated, ranging between hundreds of nanoseconds to milliseconds depending on the structure of the complex. Compared to the fast decay of endogenous autofluorescence, the transition metal characteristics can be exploited in time-gated imaging techniques, to improving signal-to-noise ratios. Lastly, the large Stokes shift of luminescent metal complexes is beneficial to avoid issues with concentration dependent quenching.

Recently, we have shown that the Re(I) complex *fac*-[Re(CO)$_3$(**phen**)(**L**^{3py})], where **phen** is 1,10-phenanthroline and **L**^{3py} is 3-pyridyltetrazolate can be utilised for live cell imaging, and it localises to acidic vesicles [17]. This complex was also found to be highly resistant to photobleaching, strongly emissive with a large Stokes shifts and long emission life times, whilst exhibiting minimal to no cytotoxicity [17]. We have also shown that changes to the tetrazolate structure result in changes in intracellular distribution, whilst retaining the key properties for live cell imaging (photobleaching resistant, large Stokes shift, minimal cytotoxicity). For example the exchange of the **L**$^{3py-}$ for 4-cyanophenyltetrazolate results in Re(I) complex (ReZolve-L1^{TM}) with shown preferential localisation within the lipid droplet [17,18]. Therefore, in the interest of designing new Re(I) complexes for cell imaging, we furthered our investigation of altering the chemical nature of the ancillary ligand to highlight consequent effects in biological behaviour of the metal-based complex. We have previously published the synthesis, electrochemical and photophysical properties of the analogous Re(I) complex bound to the 4-pyridyltetrazolate ligand, herein designated as ReZolve-ER^{TM} [19]. Remarkably, preliminary investigation into the incubation and localisation of this complex revealed a different staining pattern with respect to *fac*-[Re(CO)$_3$(**phen**)(**L**^{3py})], despite the only difference between the two complexes being the 3- or 4-pyridyl substituent. Intrigued by this initial finding we continued our investigation and here we show that ReZolve-ER^{TM}

localises to the endoplasmic reticulum (ER) [17]. Furthermore we show that ReZolve-ER™ is ideal for live cell imaging applications, with rapid detection within cells, resistance to photobleaching, consistent cellular localisation and low cytotoxicity. In addition, ReZolve-ER™ allowed the visualisation of specific nuclear events, ER structures and vesicle release from the cell surface, demonstrating that this compound is suitable for investigating a range of biological questions related to cellular dynamics.

Materials and Methods

Cell culture and staining

The non-malignant cell lines PNT1a and PNT2 and prostate cancer cell line LNCaP (clone FCG) were obtained from the European Collection of Cell Cultures via CellBank Australia (Children's Medical Research Institute, NSW, Australia). Prostate cancer cell line DU145, was obtained from the American Tissue Culture Collection via Cryosite (Cryosite Ltd., New South Wales, Australia). Chinese hampster ovaries CHO-K1 cell line and Human monocytic leukemia THP-1 cell line were obtained from the American Type Culture Collection via Sigma-Aldrich (Sigma-Aldrich, St. Louis, USA).

The PNT1a, PNT2, CHO-K1 and THP-1 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 culture medium (Sigma-Aldrich, USA), supplemented with 10% foetal calf serum (In Vitro Technologies, Australia), 2 mM L-glutamine (Sigma-Aldrich, USA). The DU-145 cell line was cultured in minimum essential medium (Gibco, Life Technologies, USA), supplemented with 10% foetal calf serum, 2 mM L-glutamine and 1 mM sodium pyruvate (Sigma-Aldrich, USA). The LNCaP cell line was cultured in RPMI-1640 media (Gibco, Life Technologies, USA) supplemented with 2 mM L-glutamine, 10 % FCS, 10 mM HEPES (Sigma-Aldrich, USA) and 1 mM sodium pyruvate. THP-1 monocytes were differentiated into macrophages by incubation with RPMI-1640 medium containing 5 ng/mL of phorbol 12-myristate 13-acetate (Sigma-Aldrich, USA) over 48 hours. Cells were cultured to approximately 90% confluence before passage, by washing with sterile PBS (Sigma-Aldrich, St. Louis, USA), TrypLE™ Express (Gibco, USA) to dissociate the cells from the culture surface and then resuspended in supplemented culture medium. Cell lines were maintained at 37°C and 5% CO_2 in a Sanyo MCO-17AI humidified incubator (Sanyo Electric Biomedical, Japan). For imaging experiment cells were culture in Ibidi µ-slides 8 wells (Ibidi, Germany) and for MTS assays PNT2 cells were cultured in 96 well plates.

For cellular imaging, cells were incubated with 50 µM ReZolve-ER™ prepared in serum free cell culture media, from a 10 mM stock solution prepared in DMSO. Cells were then imaged immediately or following 15 min of incubation without washing cells. For co-staining experiments cell were stained with ER-Tracker® Red, MitoTracker® Red CMXRos, LysoTracker® Red DND-99 or CellMask™ (Molecular Probes, USA), according to the manufactures instructions. Cells were then washed 2 x 30 sec in PBS. Serum free media containing 50µM of ReZolve-ER™ was then added and images were collected after 15 minutes of incubation. Cell fixation was performed using 4% paraformaldehyde in PBS for

20 minutes at room temperature. Cells were then washed for 3 x 10 minutes in PBS before incubation with 50 μM ReZolve-ER™ for 20 minutes.

Confocal imaging and analysis

For confocal imaging cells were held in a Uno-Combined-Controller, CO_2 microscope electric top stage incubation system (Okolab, Italy) held at 37°C and 5% CO_2. Confocal imaging was performed on a Nikon A1$^+$ confocal microscope, fitted with a LU-N4/LU-N4S 4-laser unit (405 nm, 488 nm, 561 nm, 640 nm), the A1-DUG GaAsP Multi Detector Unit (2 GaAsP PMTs + 2 standard PMTs) and a 32 channel spectral detector (Nikon, Japan). Images were captured using a 60x oil emersion lens. Each confocal micrograph represented 0.5 μm thin optical sections.

To assess emission intensity of ReZolve-ER™ over time NIS-Elements software (Nikon, Japan) was used. Regions of interest were selected using the AutoDetect function to select cells containing ReZolve-ER™, mean emission intensity was then measured over time for each region of interest. For each experiment greater than six regions of interest were used to gain the average emission intensity in Microsoft Excel 2013 (which were then plotted against time). Co-localisation between ReZolve-ER™ and ER-Tracker® Red, MitoTracker® Red CMXRos or LysoTracker® Red DND-99 was assessed in NIS-Elements software (Nikon, Japan) using the co-localisation analysis to generate a Pearson's correlation coefficient. Cells from a minimum of 10 images for each marker were measured for co-localisation and the means were compared by ANOVA analysis in GraphPad Prism with Tukey post-hoc analysis (Prism software, version 6.01, USA).

Cytotoxicity assay

To assess cytotoxicity of the complexes, cellular NAD(P)H-dependent redox activity was measured using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS) according to the manufacturer instruction (Promega, USA). Briefly, PNT2 cells were cultured as described above in 96 well plates for 24 h. Cells were then incubated with 50 μM of ReZolve-ER™ for 1 h, 4 h, 8 h or 24 h in serum free media, for a control cells were incubate with 0.5% v/v DMSO in serum free media for the corresponding incubation time without the presence of ReZolve-ER™. Media was then removed and replaced by 120 μL of MTS and PMS in RPMI-1640 medium and allowed to incubate at room temperature for 1 h. The absorbance was then measured at 490 nm by EnSpire Plate Readers (PerkinElmer, USA).

Results and discussion

The synthesis and photophysical properties of ReZolve-ER™ have previously been reported [19]. This complex was found to be compatible with fluorescent microscopy using single photon excitation at 403 nm or two-photon excitation between 800 nm and 830 nm, making it amenable for use with a range of microscopy set ups and applications.

ReZolve-ER^{TM} cellular uptake is by passive diffusion.

For effective cellular imaging, reagents need to penetrate the cell membrane and accumulate in a cell at a high enough concentration for detection by fluorescence microscopy. To assess this, live non-malignant prostate PNT2 cells were incubated with ReZolve-ER^{TM} at 50 μM, and the emission monitored in real time over 30 minutes (Supplementary Video 1). Using a Nikon A1 microscope (equipped with a live cell incubator), the emission from ReZolve-ER^{TM} was detected within seconds after the complex was applied, using a low excitation power (403 nm excitation laser set to < 2 power setting) and a low detector sensitivity (Si PMT HV detector 180). Initially ReZolve-ER^{TM} could be detected in the cytoplasm, but within the first minute of addition, the accumulation of the complex could be detected in the peri-nuclear region. The emission intensity increased throughout the cell over the first 10 minutes. After this time, the intensity and localisation of the complex appeared to be consistent for the next 20 minutes of image collection. The ability to detect the complex in cells within seconds of addition, at low laser power, is important as this will prevent photo-damage of live cells and suggested the potential for ReZolve-ER^{TM} as a real time imaging agent.

The rapid entry of ReZolve-ER^{TM} into PNT2 cells suggested that this complex was able to freely transit across the cell membrane. Furthermore, the signal from the ReZolve-ER^{TM} was concentration dependent, and a concentration of 50 μM was optimal for imaging, with lower concentrations resulting in weaker signal detection. When cells that had been labelled with ReZolve-ER^{TM} were washed (i.e. media containing ReZolve-ER^{TM} was replaced with fresh culture medium containing no dye), there was an immediate reduction in the ReZolve-ER^{TM} detection. This suggested that ReZolve-ER^{TM} had a low affinity for its cellular target; and supported the premise that a passive diffusion mechanism was involved for ReZolve-ER^{TM} cell entry, as this mechanism is dependent on a concentration gradient. To further confirm this mode of entry, cells were fixed in paraformaldehyde and incubated with ReZolve-ER^{TM}. The complex could be easily detected in these cells following fixation, confirming passive transport as the mode of cell entry (Supplementary figure 1). The rapid uptake of ReZolve-ER^{TM} is ideal for real time imaging of cells and the ability to control the addition or removal of the complex may be well suited to the long term monitoring of specific cellular structures.

To assess the photostability of ReZolve-ER^{TM} in cells, PNT2 cells were incubated with the complex and imaged continuously using a higher laser power, in an attempt to induce photobleaching. Images were collected with a scan rate of 0.22 seconds and a pixel dwell time of 0.22 milliseconds for a total of 448 scans (approximately 29 minutes), with the excitation power set to a setting of 10, which was five times greater than the maximum power required for ReZolve-ER^{TM} visualisation. Over the first eight minutes of image collection the intensity increased as the complex accumulated in cells (Figure 1), as was observed when a lower laser power was used (Supplementary video 1). Between eight and 29 minutes the emission intensity remained constant (Figure 1); suggesting that the complex was highly resistant to photobleaching. However, an increase in emission intensity was observed in the final minutes of acquisition when using a high laser power (five times the power required for visualisation), indicating that photo-activation may be occurring.

ReZolve-ER™ is detected at the ER and nucleoplasmic reticulum.

In different cell lines, including non-malignant prostate cells (PNT2 and PNT1a), malignant prostate cancer cells (LNCaP and DU145), CHO-K1 cells and THP-1 macrophages (Supplementary Figure 2), ReZolve-ER™ detected a central structure within the nucleus that resembled the nucleolus, with projections towards the nuclear membrane; as well as a diffuse reticular network emanating from the nuclear region, which extended into distal regions of the cell. To further define this intracellular distribution, ReZolve-ER™ was co-stained with ER-Tracker® (ER), MitoTacker® (mitochondria), LysoTracker® (lysosomes/acidic vesicles) and CellMask™ (plasma membrane) in PNT2 cells. PNT2 cells were labelled with the latter commercial dyes, the cells were briefly washed and then incubated with 50 μM ReZolve-ER™ for 15 to 20 minutes before imaging (Figure 2). ReZolve-ER™ showed significant co-localisation with ER-Tracker®, with a Pearson's correlation coefficient of 0.84±0.01. Co-localisation between ReZolve-ER™ and ER-Tracker® was observed on the reticular network extending from the nucleus into the cytoplasm, at the nuclear membrane and on membranous structures extending into the nucleus that appear to be nucleoplasmic reticulum (Figure 2A). The nucleoplasmic reticulum has been previously visualised using ER markers, such as ER-Tracker® [20, 21] or ER associated Ca2+-ATPase [22], which was consistent with ReZolve-ER™ detecting a similar, biologically related structure. In contrast, there was only a limited amount of ReZolve-ER™ detected in association with mitochondria (Figure 2B) and lysosomes (Figure 2C), which was shown by lower Pearson's correlation coefficients for co-localisation between ReZolve-ER™ and either MitoTracker® or LysoTracker® of 0.44±0.01 or 0.37±0.01, respectively. While most of the mitochondrial and lysosomal labelling was independent of ReZolve-ER™ some overlap was detected between these structures (Figure 2B, 2C). This overlap between ReZolve-ER™ and mitochondria or lysosomes was not surprising given the close association between the ER and these two subcellular compartments [23, 24]. Interestingly, while ReZolve-ER™ did not stain large areas of the plasma membrane (Figure 2; i.e. limited co-localisation with CellMask™), there was some specific sites of co-localization with CellMask™ (Figure 5B), which may have identified a localised interaction between the endoplasmic reticulum and the cell surface.

From this co-location study we propose that ReZolve-ER™ locates to the ER and biologically related structures, in live cells. The mechanism by which ReZolve-ER™ associates with the nuclear membrane/ER is not currently known, but a similar tricarbonyl rhenium(I) diimine luminescent complex, ReZolve-L1™, has been shown to localise with polar lipids in cells [17, 18]. Given that the ER is a major site of lipid synthesis [25], we can speculate that ReZolve-ER™ may have a similar lipophilic interaction.

ReZolve-ER™ remains in ER structures during longer term real time imaging.

For long term imaging experiments it is important for a reagent to be retained in cells over a long period of time, and to cause minimal to no damage to the cells. PNT2 cells were incubated with ReZolve-ER™ and imaged for 280 minutes at 10 minute intervals to assess the compatibility of the complex with long term imaging. Over 280 minutes ReZolve-ER™ remained localised to the perinuclear region of cells (Figure 3A-C). Although the intensity of

the emission from ReZolve-ER™ decreased over time, it was still easily detected at the end of the time course (Figure 3C, 3D). The decrease in emission intensity was likely due to the complex being slowly trafficked out of the cell, and not related to photobleaching, as the complex was photo-resistant and exhibited continuous emission in response to extended laser exposure (Figure 3). To assess the potential cytotoxic effects of ReZolve-ER™, PNT2 cells were incubated with the complex for 1, 4, 7 or 24 hours and the cell viability assessed via an MTS assay (Figure 3E). Cell viability (as indicated by absorbance) was reduced when treated with ReZolve-ER™ at 50 μM for 1 hour and 4 hours compared to controls, however the viability was still significantly higher than the negative control (Figure 3E). Although cell viability was reduced by this complex, cells imaged with ReZolve-ER™ for the 4 hours did not show morphological changes, which would have indicated cytotoxicity during this time, thus suggesting that the cytotoxicity of ReZolve-ER™ was low. Interestingly, following 7 hours and 24 hours of incubation with ReZolve-ER™ the cell viability was unchanged when compared to controls (Figure 3E). While this may be due to complex efflux, it showed that long term exposure to the ReZolve-ER™ does not affect overall cell survival. The minimal cytotoxic effects of ReZolve-ER™ and its ability to be detected in cells over a long time periods make this complex an ideal tool for live cell imaging.

Detection of nuclear and ER events with ReZolve-ER™.

A number of time series images were collected using ReZolve-ER™ (Figures 4 and 5) and these revealed inward projections from the nuclear membrane, which resembled a phagocytic event at the nuclear membrane (Figure 4), as well as a number of small vesicles in the cellular periphery (Figure 5). Figure 4 shows that ReZolve-ER™ detected the formation of a large ~3 μm nuclear membrane derived phagosome that appeared to sequester part of the nucleoplasm (Supplementary Video 2). ReZolve-ER™ clearly defined the nuclear membrane extension as well as the phagosome vesicle formation, closure and release event, which was evident during the 5 minute imaging time course. It is generally accepted that trafficking into and out of the nucleus occurs through nuclear pores, which is facilitated by the nuclear pore complex [26], but budding and formation of vesicles can also occur from the nuclear envelope [27-29]. For example, ribonucleoprotein particles can be exported out of the nucleus in a process similar to viral capsid nuclear egress, where small vesicles have been observed forming at the nuclear envelop, before budding out to release their content [27]. In TEM images multivesicular bodies have also been visualised in close proximity to the nuclear envelope and in some cases these multivesicular structures appeared to be forming from the nuclear envelope [28,29]. Although the identity of this nuclear structure is unclear, and we cannot rule out an artificially induced cellular response to the complex, it seemed that ReZolve-ER™ was able to visualise specific nuclear events in real time, indicating its potential for live cell imaging applications.

To further explore the detection of small (< 0.5 μm) vesicles by ReZolve-ER™ in the cellular periphery, a dual labelling time-course experiment was performed using both ReZolve-ER™ and CellMask™. This enabled the simultaneous visualisation of two distinct vesicular events at the cell surface (Figure 5; Supplementary Video 3). CellMask™ identified a ~ 1 μm diameter vesicle forming over an eight minute time course, involving a membrane protrusion

from the cell surface and then vesicle budding/excision from the plasma membrane; and interestingly this microvesicle contained a small amount of diffuse ReZolve-ER^{TM} staining in its lumen (Figure 5B). The detection of ReZolve-ER^{TM} inside the budding vesicle (Figure 5B) could be consistent with the dissociation of the dye from the membrane into the vesicle (N.B. cell washing significantly reduced the staining, suggesting that ReZolve-ER^{TM} has a low affinity for its target); however we could not exclude the possibility that ReZolve-ER^{TM} was identifying a specific target within these budding vesicles. In addition, a small (< 0.5 μm) vesicle with intense ReZolve-ER^{TM} staining was visualised: first distorting a specific location on the plasma membrane and showing co-location with CellMask™; and then pushing through the plasma membrane to be released from the cell (Figure 5C). This later event occurred more rapidly (~ 2-3 minutes) when compared to the former 1 μm diameter vesicle formation and release (~ 8-9 minutes). These observations suggest that ReZolve-ER^{TM} has the potential for not only visualising the ER, but also vesicle trafficking out of the cell.

Conclusion

Visualising cellular organelles such as the ER can provide powerful insights for understanding cellular dynamics under a range of physiological conditions. The ER plays a central role in protein transcription, molecular trafficking and cellular signalling. Endoplasmic reticulum stress is commonly observed in a range of diseases and in response to physiological stress, and thus the ability to track the ER over large time periods may provide new insights into diseases such as cancer, non-alcoholic fatty liver disease, diabetes and neurodegenerative disease. Thus an imaging reagent such as ReZolve-ER^{TM} that can monitor the ER has potential applications in facilitating our understanding of cell biology in a range of diseases. Given that ReZolve-ER^{TM} has the ability to be used over short or long time courses, is highly resistant to photobleaching, can be removed from cells, has low cytotoxicity over long exposure times and has already demonstrated its ability to detected interesting cellular phenomena in real time, this imaging reagent could be utilised in a multitude of experimental protocols and provide a flexible imaging tool for cell biologists.

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Author contribution

CB, SP, MM and DB conceived and supervised the study; CB SP and DB designed experiments; CB and AS performed experiments; PS, PW and SS provided and characterised new tools and reagents; CB, SP and DB wrote the manuscript; AS, PS, PW, SS and MM made manuscript revisions.

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Figures

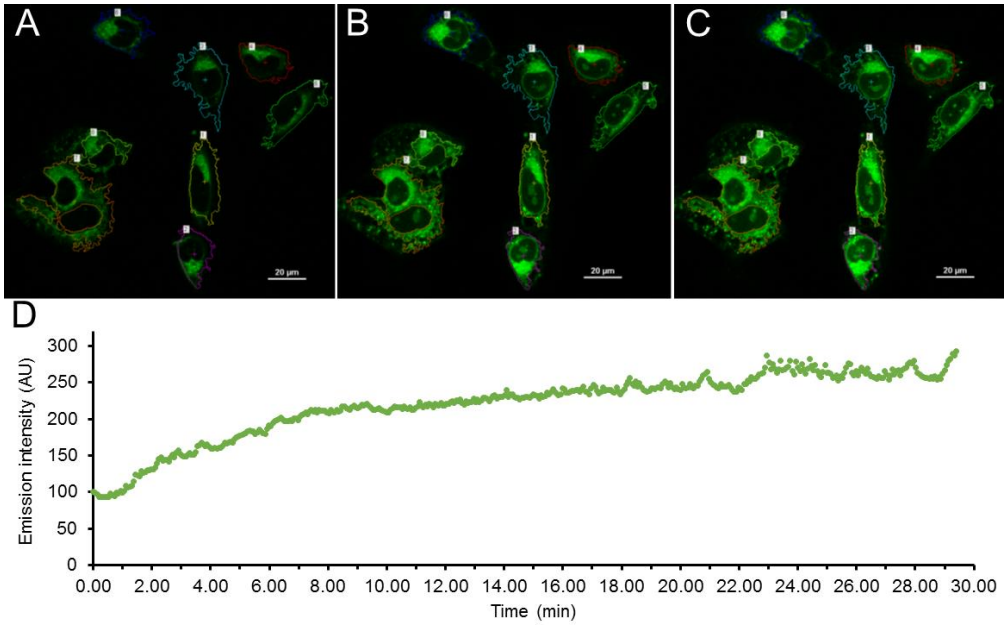

Figure 1. ReZolve-ER™ does not photobleach when expose to high laser power. *(A-C) Confocal micrographs of PNT2 cells incubated with ReZolve-ER™ for 0 min (A), 15 min (B) or 29 min (C). Overlay shows the regions of interest from which emissions intensity was measured. (D) Scatter plot showing average emission intensity (au) of ReZolve-ER™ stained cells from regions of interest indicated. Images collect with 403 nm excitation set to laser power 10, a scan rate of 0.22 s (give a total of 448 scans over 29.33 min) and a pixel dewell time of 0.22 ms.*

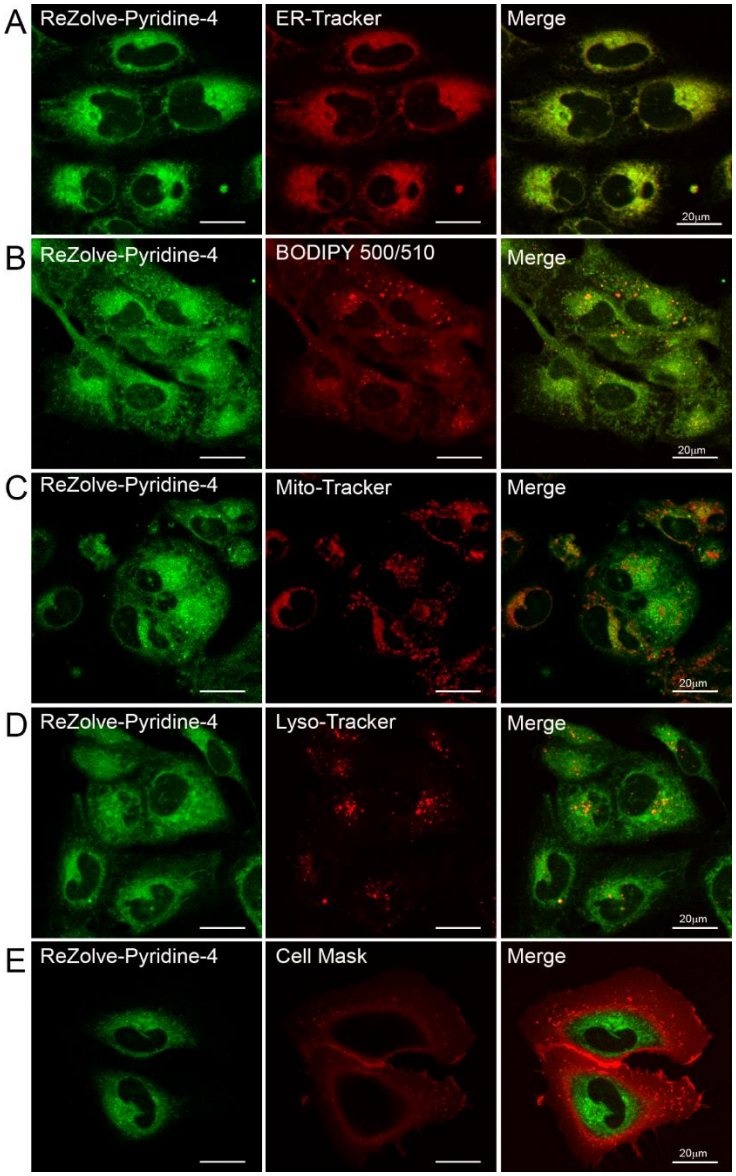

Figure 2. ReZolve-ER™ subcellular localisation. *Confocal micrographs showing PNT2 cells incubated with ReZolve-ER™ (green) and counter stained with (A) ER-Tracker® for the labelling of ER (red), (B) MitoTracker® for the labelling of mitochondria (red), (C) LysoTracker® for the labelling of lysosomes/acidic compartments (red), and (D) CellMask™ for the labelling of the plasma membrane (red).*

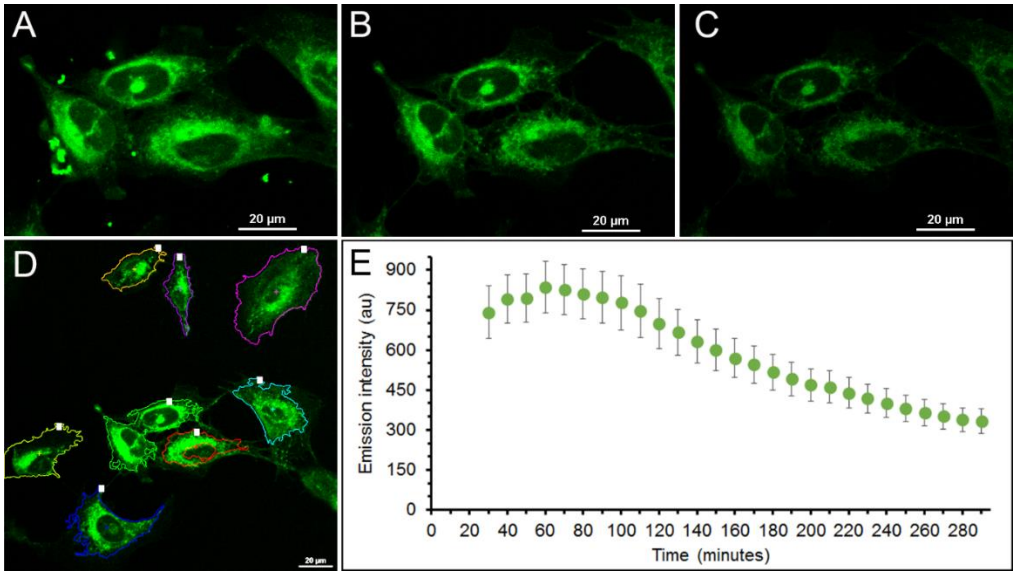

Figure 3. ReZolve-ER™ localisation is unchanged over time.

*(A-D) Confocal micrographs of PNT2 cell incubated with ReZolve-ER™. Images taken at 20 min (A), 140 min (B) and 290 min (C) incubation time points. (D) Shows a dot plot of emissions intensity over time. (E) Histrogram of cell viability measured by an MTS assay in response to incubation with ReZolve-ER™ (Re-ER) at 50µM for 1 h, 4 h, 7 h or 24 h, when compaired to a negative control of 50% DMSO in culture media (-ive control), a positive control of cells will full media (+ive control), and time point controls (control) in which cells were expose to 0.5% DMSO for 1 h, 4 h, 7 h or 24 h. * indicates a significant difference between the negative control and all other treatment conditions, ** indicates significant difference between indicated groups.*

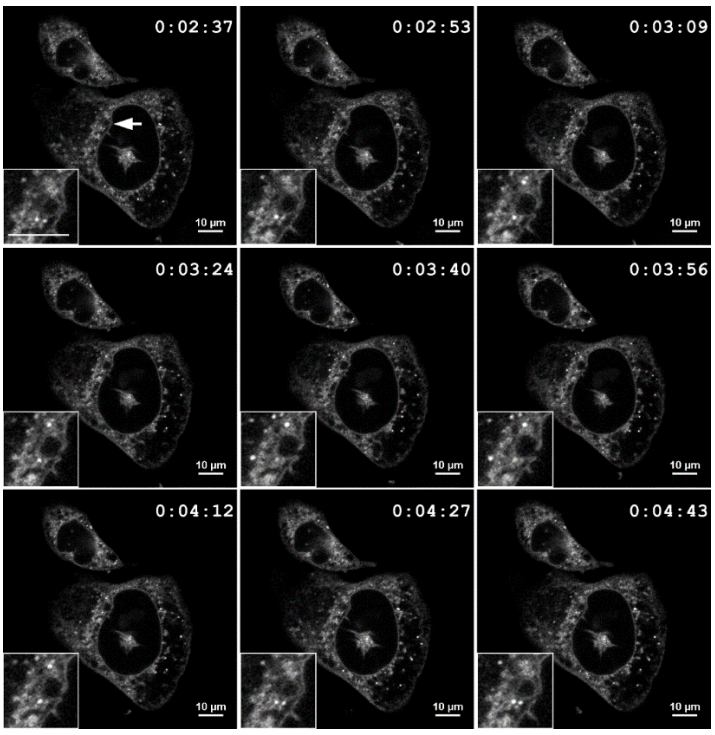

Figure 4. Time lapse confocal imaging of ReZolve-ER™ in PNT2 cells. *Time of image capture is indicated in the right hand corner of each frame; and represents the last five minutes in a set of three consecutive time courses (i.e. total 15 min). Scale bar = 10 µm. Enlarged panel shows nuclear phagosome (~ 3 µm) forming over the time course and intense small (< 0.5 µm) vesicular staining.*

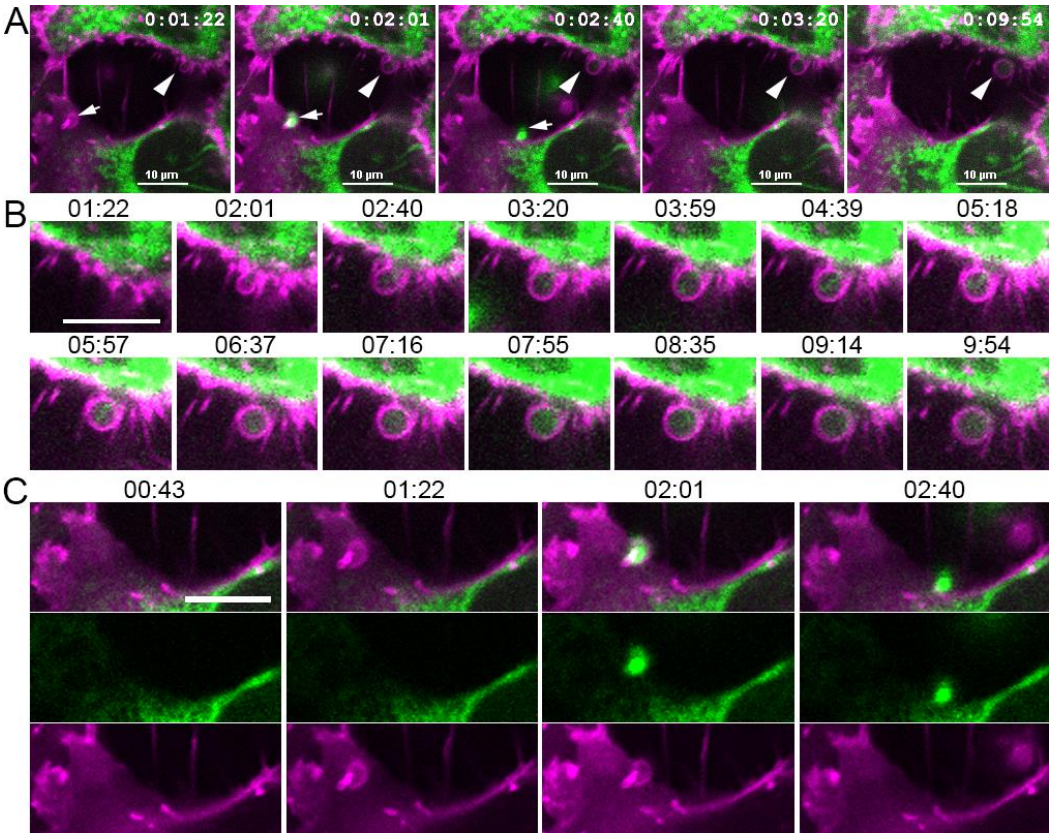

Figure 5. ReZolve-ER™ imaging of vesicle release from the cell surface. *Time lapse confocal micrographs showing PNT2 cells stained with ReZolve-ER™ (green in A, B and C), counterstained with Cell Mask (purple in A, B and C). (A) Two vesicle release events were captured, arrow head indicated cross-section of budding vesicle forming which is enlarged in panel B; arrow indicates budding event at the cell surface which is enlarged in panel C. Time of capture is indicated in the right hand corner of each frame (A) or above each frame (B and C). Scale bar =10 µm.*