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Biosensors for Monitoring Autophagy

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1. Introduction

1.1 Prologue

Autophagy ("self-eating") is a highly conserved intracellular degradation process. Recently, an astonishing number of connections to human physiology and disease for autophagy have been made. Thus, autophagy plays a critical role during development and differentiation, in cancer, neurodegeneration, combating viral and microbial infections, and has been linked to life span extension and ageing. For these reasons, the ability to follow autophagy in living mammalian cells is of particular interest, both in terms of developing better understanding at a mechanistic level and in terms of possible future clinical applications.

In this chapter our intention is not to summarise the available autophagy assays, but to highlight the application of biosensors used to monitor autophagic processes in live cells. Firstly, we briefly outline current knowledge about the molecular mechanism and function of autophagy (Section 1.2). Then, we outline how autophagy can be measured in live cells in a non-invasive manner and indicate some of the advantages and disadvantages of the biosensor-based assays of autophagy currently in use (Section 2). In particular we focus here on the design and use of Rosella - a dual wavelength emission biosensor based on a fusion of fluorescent proteins whose use exploits alterations in pH during autophagy. The advantages of this approach will be highlighted (Section 2.3) and illustrated by applications to follow autophagy both in yeast, an important eukaryotic model organism (Section 2.3.1) and mammalian cells (Section 2.3.2). Finally, we address the future prospects for application of alternative approaches (Section 3) to the measurement of autophagy based on the use of novel probes.

1.2 Autophagy - different pathways, mechanism and selectivity

The biological importance of autophagy is such that the molecular mechanism is the focus of intense research efforts (reviewed in Yang & Klionsky, 2010). Three distinct autophagic pathways have been shown to operate in eukaryotic cells namely, macroautophagy, microautophagy and chaperone-mediated autophagy-CMA (Orenstein & Cuervo, 2010). In order to understand the rationale of biosensor design and function it is necessary to have a basic understanding of the particular process to be monitored. The key aspects of each of the three different forms of autophagy are shown in **Fig. 1**.

The morphological hallmark of macroautophagy (hereafter referred as autophagy) is the formation of a double-membrane structure called an autophagosome, encapsulating the

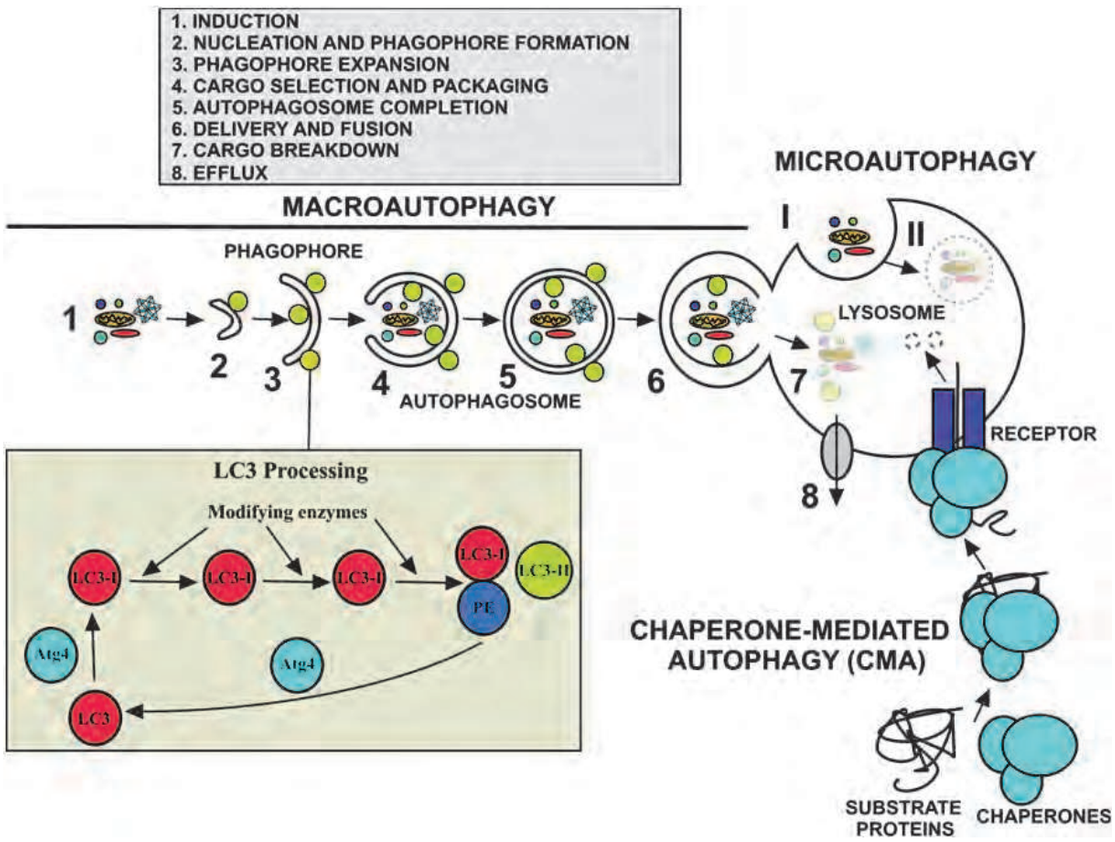

Fig. 1. Three main types of autophagy.
Three morphologically and mechanistically distinct types of autophagy have been described in cells: macroautophagy, microautophagy and chaperone-mediated autophagy (CMA). Microautophagy is best described in yeast cells and CMA is restricted to mammalian cells. The degradative vacuole in yeast cells is large, whereas the lysosomes are much smaller and far more numerous. In this diagram the lysosome is not drawn to scale in order to illustrate the three processes that deliver material to it. Microautophagy can be considered a two-step process: lysosomal membrane invagination (I) followed by cargo breakdown (II). The individual steps of macroautophagy are numbered (see shaded box for key). Inset (LC3 processing) summarises the steps leading to the recruitment of LC3-II (green spheres) to the forming autophagosome. Completed autophagosomes fuse with lysosomes, after which LC3-II on the cytoplasmic face of autolysosomes is delipidated through the activity of Atg4 and recycled. LC3-II located on the internal surface of autophagosomes is degraded in the autolysosomes. The association of LC3-II with autophagosomes has been widely exploited as a marker for autophagy. LC3, microtubule-associated protein 1 light chain 3; PE, phosphatidylethanolamine.

material for degradation (reviewed in Xie & Klionsky, 2007). The pathway consists of a number of distinct steps and includes: (1) induction, (2) nucleation and phagophore formation, (3) phagophore expansion, (4) cargo selection and packaging, (5) autophagosome completion, (6) cargo delivery and fusion of the autophagosome with the lysosome/vacuole, (7) breakdown of the sequestered cargo and (8) efflux (reviewed in Lagakis & Klionsky, 2006) **(Fig. 1)**. Upon autophagy induction, that is tightly regulated by Tor (target of

rapamycin kinase), a double membrane structure termed the phagophore is initiated and expands to engulf the cargo. The sequential expansion of the phagophore allows the cell to adjust the size of the autophagosome to sequester cellular components over a wide size range and can include organelles such as a mitochondrion (reviewed in He & Klionsky 2009; Kanki & Klionsky, 2010). The ends of the phagophore membrane fuse to form the autophagosome, thereby, sequestering the cargo from the rest of the cell. The outer-membrane of the autophagosome then fuses with the membrane of the lysosome (mammalian cells) or the vacuole (yeast cells) to deliver the cargo into to the acidic lumen (~pH 4.5-6). Cargo is degraded through the action of resident hydrolases to basic polymer building blocks (e.g., nucleotides, amino acids, sugars) which are exported to the cytosol for recycling by the cell (reviewed in Yorimitsu & Klionsky, 2005).

Autophagy is regulated by the autophagy-related genes (*ATG*) together with some other particular gene products required for selective sequestration and degradation of specific cargos (see below). Among the 35 *ATG* genes identified through pioneering studies in yeast, 15 are required for core autophagy functions, while others play particular roles in one or more types of autophagy (reviewed in Kanki & Klionsky, 2010). Homologues for many of these genes (~15) have been identified in mammals (Tanida, 2010; Tanida, 2011). Although the order of action and interactions between *ATG* gene products have been determined (reviewed in Legakis & Klionsky, 2006; Chen and Klionsky, 2011) few of the proteins encoded by the *ATGs* have motifs that provide insights into their function, and the precise mechanism of action of many remains unknown.

Nevertheless, details of how a number of individual gene products function in the process is emerging. Some of the gene products act sequentially in two ubiquitin-like conjugation systems (Atg8 and Atg12) that are involved in vesicle expansion and completion (Chen & Klionsky, 2011). *ATG8* and *ATG4* are of particular significance as their gene products have been exploited in a number of assays for autophagy (Section 2.2). In mammals the equivalent genes (of which there are several) encode LC3 (microtubule-associated protein 1 light chain 3) and autophagins (e.g., Atg4b), respectively (Tanida, 2010). LC3 in mammalian cells is immediately modified post-translation by the cysteine protease activity of an autophagin to form LC3-I, a soluble protein found throughout the cytosol. When autophagy is induced LC3-I is lipidated by the addition of PE (phosphatidylethanolamine) to form LC3-II and associates with the phagophore membrane. After completion of the autophagosome and during fusion with the vacuolar/lysosomal membrane, LC3-II is recycled from the outer-membrane by the action of an autophagin. Since LC3-II remains on the inner autophagosome membrane, when fused to GFP it can be used to track fusion of autophagosomes with the lysosomes, if the latter are appropriately labelled **(Fig. 1; Inset)** (reviewed in Mizushima et al., 2010).

Autophagy plays a role in the degradation of a wide range of cellular components, including long-lived proteins, protein complexes and aggregates, macromolecules (ribosomes, lipids), organelles (the endoplasmic reticulum, peroxisomes, the nucleus and mitochondria) and even intracellular pathogens (bacteria and viruses) (reviewed in Klionsky et al., 2007a). Although starvation-induced autophagy is thought to act non-selectively in the degradation of bulk cytoplasm, it can be highly selective in cargo selection. Protein aggregates (Rubinsztein, 2006), nuclear components (Krick et al., 2008; Roberts et al., 2003) or superfluous, damaged/stressed or nutritionally-challenged organelles, such as mitochondria (Kanki et al., 2011; Youle & Narendra, 2011) may be targeted. Selective autophagy is best characterised in yeast (reviewed in Farré et al., 2009; van der Vaart et al.,

2008) and includes other modes not described in mammalian cells such as the cytoplasm to vacuole targeting (Cvt) pathway (reviewed in Lynch-Day & Klionsky, 2010) and the vacuolar import and degradation (vid) pathway (Brown et al., 2010).
Cargo sequestration can take place through microautophagy, but the limiting/sequestering membrane in this case is the vacuolar/lysosomal membrane itself, which invaginates to form tubules or vesicles that pinch off into the lysosomal lumen **(Fig. 1)**. Our current understanding of microautophagy mechanism and regulation is largely based on studies in yeast. Microautophagy remains poorly understood in mammals (Mijaljica et al., 2011; Shpilka & Elazar, 2011).
Delivery of cargo via CMA does not require formation of intermediate vesicle compartments or membrane fusion. Instead, the substrates, soluble proteins, are translocated from the cytosol into the lysosomal lumen directly across the membrane in a process mediated by a translocation protein complex that requires activity of chaperones, substrate unfolding and the presence of receptors on the lysosomal membrane **(Fig. 1)**. CMA occurs only in mammalian cells (reviewed in Orenstein & Cuervo, 2010).

2. Approaches for monitoring autophagy

"Static measurements" of a particular biological process can be limiting in terms of the insights they provide. Autophagy is a complex, highly regulated and dynamic process involving membrane rearrangement events including formation of the autophagosome and fusion of intracellular membranes. Monitoring such dynamic events represents a challenge and biosensors specific for many of the selective forms of autophagy in particular are yet to be developed. The lack of suitable biosensors likely underlies many of the misconceptions in our understanding of the function of autophagy, mammalian autophagy in particular.
Nevertheless, new techniques have been developed both to monitor autophagy as a dynamic process and to modulate autophagy to facilitate probing its function in a given cellular process (reviewed in Klionsky et al., 2008; Mizushima, 2004; Mizushima et al., 2010; Mizushima & Yoshimori, 2007; Rubinsztein et al., 2009). Key components of the molecular mechanism have been identified, many of which have the potential to be exploited as useful reporters **(Fig. 1)**.
As interest in selective modes of autophagy gains momentum, it is increasingly important to have access to probes able to follow events in a selective manner. Since it is likely that some cellular targets are present in only small quantities, biosensors that allow monitoring of autophagy at low levels are required. Such a biosensor is also likely to facilitate the detection of autophagy at early stages after induction. It is often important to follow biological processes in living cells, tissues or organisms requiring the application of non-invasive techniques. Such applications are based commonly on the use of light-based probes that rely on the chemical production of light, or are based on fluorescence emission. Autophagy is a temporally and spatially dynamic process; therefore, it is desirable that biosensor outputs do not persist or accumulate in a way that would prevent successive cycles of activity to be detected. A single ideal biosensor fulfilling each of these requirements has yet to be developed.
Biosensor-based approaches for monitoring autophagy can be considered to fall into either of two major categories **(Fig. 2)**, examples of which have been reported in the literature (reviewed in Klionsky et al., 2007b; Klionsky et al., 2008; Mizushima et al., 2010). In the first category, autophagic activity can be assessed by monitoring the behaviour, modification or activity of key molecules involved in the molecular mechanism of autophagy (Section 2.2).

Alternatively, the fate of the target material itself can be followed either by monitoring delivery to the lysosome/vacuole, or its degradation (Section 2.3). In order to follow the fate of the target material itself, it is crucial to be able to identify or selectively label the target. This can be achieved to a limited extent by visualising characteristic staining patterns of particular targets such as mitochondria or intracellular pathogens using electron microscopy (Klionsky et al., 2007b; Klionsky et al., 2008; Mizushima et al., 2010), or by labelling with dyes suitable for detection by fluorescence microscopy. The scope for labelling autophagic targets is greatly expanded if genetically encoded probes such as fluorescent proteins (FPs) are considered. Genetic fusions of target proteins with FPs can be readily expressed in cells allowing the delivery of targets to the lysosome or vacuole to be tracked using fluorescence microscopy in individual cells. FPs are remarkably stable molecules and persist as

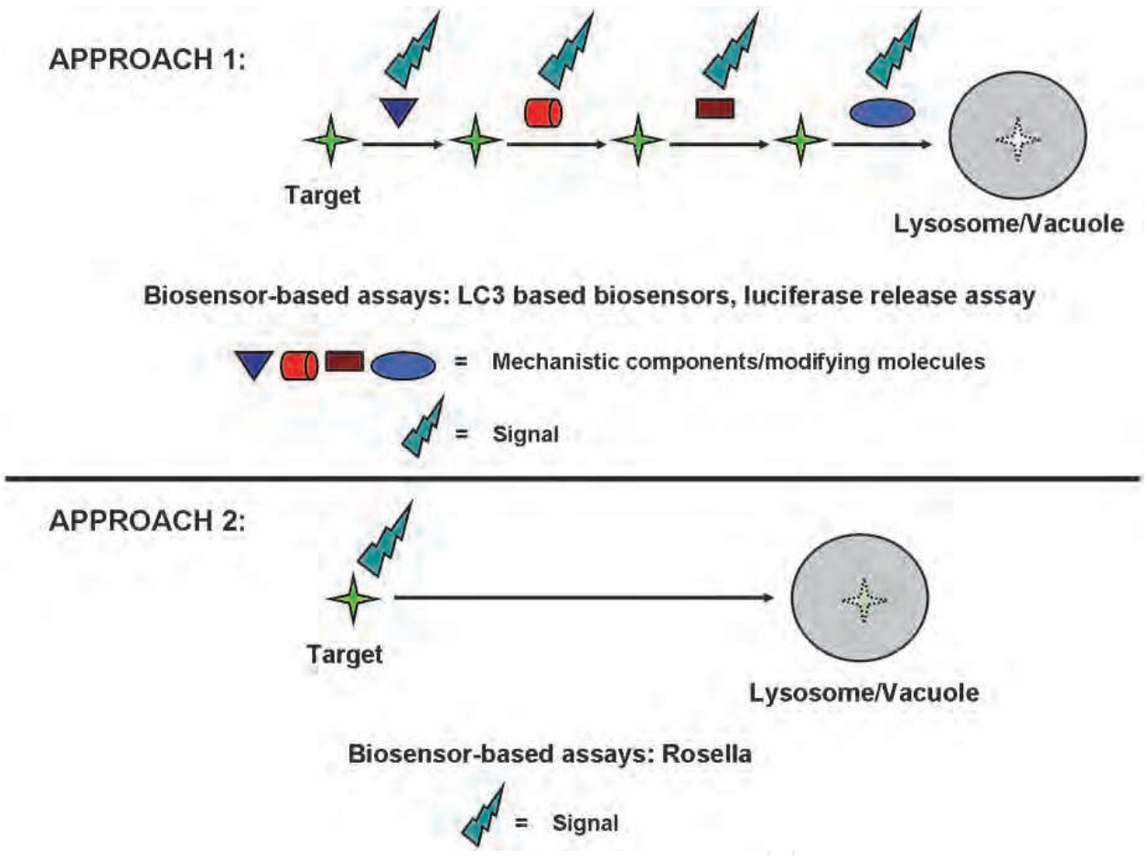

Fig. 2. Approaches for monitoring autophagy.

The molecular mechanism of autophagy may be exploited to monitor the process by tracking the modification or activity of key molecules in the pathway (Approach 1). For example, post-translational modification of LC3 or its recruitment to the autophagosome is commonly used. This approach does not provide information on the identity of the cargo, which must also be labelled if selective forms of autophagy are to be tracked. Alternatively, (Approach 2) labelling the target with a biosensor (e.g., Rosella) that continually senses and reports on the environment of the target material allows for monitoring of selective autophagy. However, this approach does not provide information on the mechanistic aspects of the particular process involved.

fluorescent forms in the degradative organelle long enough to detect their presence (Katayama et al., 2008). Furthermore, the fate of such genetic fusions can be followed in populations of cells using western blots to follow changes in sizes of the fusion produced by proteolytic degradation within the degradative organelle (Mizushima et al., 2010; Klionsky et al., 2008). In specific cases, enzymatic activities can also be used to follow delivery to the lysosome/vacuole (Ketteler et al., 2008; Ketteler & Seed, 2008; Kimura et al., 2007; Klionsky et al., 2007b; Klionsky et al., 2008; Mizushima et al., 2010). As new forms of selective autophagy are reported in the literature approaches that label and follow the target material for autophagy are becoming the focus of attention.

2.1 Fluorescence-based biosensors for monitoring autophagy in live cells

Monitoring autophagic activity or flux in cells, tissues or organs is of considerable interest to many researchers, and is a technology undergoing continual development. In recent years, methods for detection of autophagy suitable for *in vivo* applications have been developed (Ketteler & Seed, 2008; Mizushima et al., 2010). Nevertheless, ideal methods for any one particular application may not yet exist (Klionsky et al., 2008).

In the following section, we describe some of the available biosensors suitable for analysis of autophagy/autophagy-related pathways *in vivo*. These biosensors exploit fluorescent proteins or luciferase as reporters to generate a readily detectable light signal. The first category (as discussed above) exploits Atg8/LC3 and autophagin Atg4b (Section 2.2), key proteins in the molecular mechanism, whilst the second relies on direct labelling of the autophagic cargo (e.g., mitochondria, the nucleus) using the Rosella biosensor (Section 2.3).

2.2 LC3

LC3 (or Atg8 in yeast), fused at its N-terminus to one of a number of different colour FPs including GFP, CFP (Ravikumar et al., 2010) or mCherry (Axe et al., 2008; Klionsky et al., 2008; Mizushima et al., 2010), has been used with great success to monitor autophagy in a number of different model organisms including *Saccharomyces cerevisiae* (Klionsky et al., 2008; Xie et al., 2008), *Caenorhabditis elegans* (Klionsky et al., 2008; Meléndez et al., 2003), *Dictyostelium discoideum* (Klionsky et al., 2008; Otto et al., 2003), *Drosophila melanogaster* (Klionsky et al., 2008; Rusten et al., 2004; Scott et al., 2004), *Arabidopsis thaliana* (Klionsky et al., 2008; Yoshimoto et al., 2004), zebrafish (He et al., 2009) and mice (Mizushima et al., 2004; Mizushima et al., 2010). Transgenic mice expressing GFP-LC3 and mCherry-LC3 in a systemic or tissue-specific manner have been used to show alterations in autophagic activity under disease and stress conditions (Iwai-Kanai et al., 2008; Mizushima et al., 2010). LC3-based approaches underpin assays in current use to measure autophagic flux (Mizushima et al., 2010).

When viewed using fluorescence microscopy, the diffuse cytosolic GFP-LC3-I is converted to GFP-LC3-II upon induction of autophagy, which subsequently associates with the phagophore membrane and appears as bright fluorescent puncta in the cytoplasm. The number of puncta in a cell has been used as a measure of autophagic activity (Kabeya et al., 2000; Klionsky et al., 2008; Mizushima et al., 2010). GFP-LC3 on the outer surface of the completed autophagosome is removed by action of an autophagin just prior to its fusion with the degradative organelle. Fluorescence due to GFP-LC3 residing on the internal membrane of the autophagosome can persist in the autolysosome until degraded, dependent on the identity of the FP and pKa of its chromophore (Section 2.3).

Image based assays can be time consuming, labour-intensive and require experience for accurate interpretation. Fluorescence activated cell sorting (FACS) allows the rapid acquisition of large datasets and has been used to monitor autophagy by following the time–dependent decrease in the cellular GFP-LC3 signal as it is consumed during autophagy in live cells (Shvets et al., 2008).

Fusing LC3 to a red fluorescent protein (mRFP) and a GFP (EGFP) in tandem to form tfLC3, represents an approach that allows the environment of the probe to be monitored (Kimura et al., 2007; Klionsky et al., 2008). The fluorescence emissions of these two FPs differ in their response to pH such that mRFP remains highly fluorescent in the acidic lumen of the lysosome, whereas EGFP emission is essentially quenched. tfLC3 enables simultaneous estimation of both the induction of autophagy and flux through autophagic compartments without the need to use inhibitors and inducers of autophagy or any other drug treatment (Kimura et al., 2007).

An approach has been developed that monitors the enzymatic activity of Atg4b using LC3 fused to luciferase as a substrate (Ketteler et al., 2008; Ketteler & Seed, 2008). The luciferase is expressed fused at its C-terminus to LC3 and in turn to β-actin. Anchored to actin, the modified *Gaussia* luciferase cannot exit the cell into the growth medium unless it is first released by the action of Atg4b on the LC3 moiety, the preferred substrate (Ketteler et al., 2008). The growth medium is assayed for the presence of luciferase activity. In the future, transgenic mice expressing the luciferase release reporter system may provide convenient, tractably quantitative, mouse models of autophagy (Ketteler & Seed, 2008).

2.3 Rosella: a biosensor for selective autophagy

The growing interest in understanding the regulation and mechanism of selective forms of autophagy (reviewed in Farré et al., 2009; van der Vaart et al., 2008) demands the use of approaches that provide information on specific or individual cargos. However, each of the forgoing assays (Section 2.2) report on the activity of a component of the autophagic pathway, and require the use of additional probes in combination if individual autophagic cargo is to be followed. We have established an alternative approach that senses and reports delivery of the target material to the lysosome or vacuole **(Fig.2)**. The assay is based upon a genetically encoded dual colour-emission biosensor, Rosella, the fusion of a relatively pH-stable fast-maturing variant of the red fluorescent protein, DsRed.T3 with a pH-sensitive variant of green fluorescent protein, pHluorin **(Fig. 3A)** (Devenish et al., 2008; Rosado et al., 2008). By virtue of it being targeted to specific cellular compartments or fused to an individual protein, the biosensor provides information about the identity of the cellular component being delivered to the lysosome or vacuole for degradation. Importantly, the pH-sensitive dual colour fluorescence emission provides information about the environment of the biosensor **(Fig. 3B)** (Devenish et al., 2008; Rosado et al., 2008).

We have already shown that the Rosella biosensor can be used to follow autophagic degradation of specific cargo (cytosol, mitochondria and the nucleus) in yeast cells (Devenish et al., 2008; Mijaljica et al., 2010; Nowikovsky et al., 2007; Rosado et al., 2008) by fluorescence microscopy and FACS analysis (Devenish et al., 2008; Mijaljica et al., 2010; Rosado et al., 2008). Here, we extend those findings and demonstrate the use of Rosella to study reorganisation of the nuclear membrane in mutant yeast strains. We also show that Rosella can be used for monitoring both non-selective autophagy (cytosol) and selective autophagy (mitochondria) in mammalian cells highlighting the benefits of use of this biosensor.

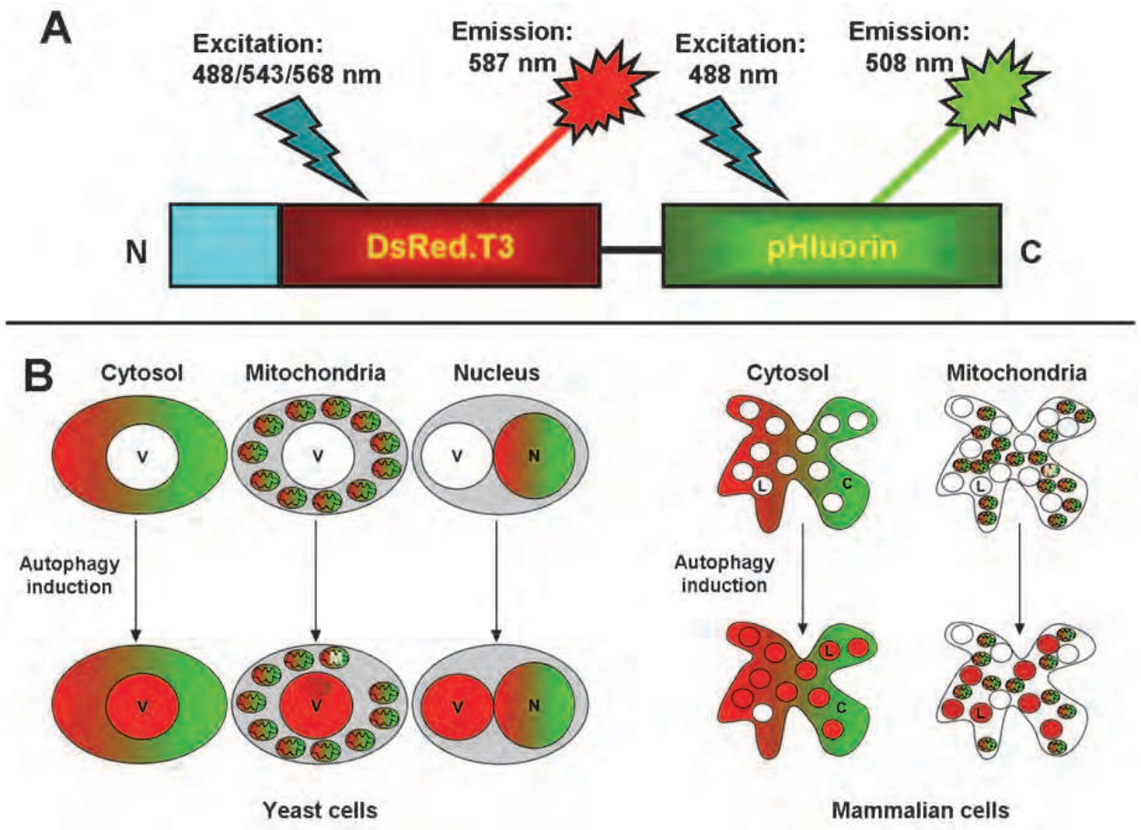

Fig. 3. Rosella can be used to monitor autophagy of different cellular compartments in yeast and mammalian cells.
(A) A schematic of Rosella. The components of Rosella are as follows: targeting sequence for subcellular localisation (cyan box) or other protein to which it is fused; red fluorescent protein (DsRed.T3); polypeptide linker (black bar) and a pH-sensitive GFP variant (pHluorin). A targeting sequence is not required for cytosolic Rosella. The wavelength of lasers suitable for excitation of each component fluorescent protein is indicated. The λ_{max}^{em} (maximum emission wavelength) of each FP is indicated.
(B) Appropriately targeted Rosella can be used to monitor delivery of cytosol, mitochondria or nucleus to the vacuole of yeast cells or cytosol and mitochondria to the lysosome of mammalian cells. When autophagy is induced, increased amounts of Rosella which fluoresces both red and green outside of the acidic vacuole or lysosome (indicated by red and green colour) accumulate in these compartments (indicated by red colour), whereby the green fluorescence emission is quenched. C, cytosol; L, lysosome; M, mitochondria; N, nucleus; V, vacuole.

2.3.1 Monitoring nucleophagy in yeast cells using Rosella

Nucleophagy in the yeast *S. cerevisiae* can occur by a microautophagic process based on the morphological distinction that the cargo destined for degradation within a nuclear bleb is directly engulfed and sequestered into an invagination of the vacuolar membrane rather than being packaged into autophagosome-like vesicles. This process has been referred to as micronucleophagy and piecemeal microautophagy of the nucleus (PMN). Upon nutrient

deprivation of cells (e.g., nitrogen starvation) PMN is initiated at nuclear-vacuolar (NV) junctions and promoted by the interaction of specific membrane-bound proteins (Krick et al., 2008; Kvam & Goldfarb, 2007; Roberts et al., 2003). PMN takes place through a series of morphologically distinct steps. First, an NV junction forms at which the nuclear envelope, coincident with an invagination of the vacuolar membrane bulges into the vacuolar lumen. Later, a fission event releases into the vacuolar lumen a nuclear-derived vesicle (PMN vesicle) filled with nuclear material enclosed by both nuclear membranes. Eventually, the PMN vesicle is degraded by resident vacuolar hydrolases (Krick et al., 2008; Kvam & Goldfarb, 2007; Roberts et al., 2003).

n-Rosella is a variant of Rosella targeted to the nucleus. Under growing conditions, wildtype yeast cells expressing n-Rosella exhibit fluorescent labelling of the entire nuclear lumen (nucleoplasm), which appears as a single red and green body **(Fig. 4)** (Devenish et al., 2008; Mijaljica et al., 2007; Mijaljica et al. 2010; Rosado et al., 2008). When incubated in nitrogen starvation medium for 24 h, cells show red and green fluorescence of the nucleus as well as markedly visible accumulation of red fluorescence in the vacuole indicative of autophagy (nucleophagy) **(Fig. 4A)**.

n-Rosella labelling allows both the morphology of the nucleus to be readily visualized and its own accumulation inside the vacuole. The biosensor can also be used to monitor intermediate steps in the process, using yeast cells lacking expression of particular *ATG* genes (**Fig 4B**). In this example blebbing of the nucleus into the vacuole can be seen. Since the bleb remains both red and green fluorescent, we can conclude that the bleb has a relatively high pH, and the membrane structures required to isolate the vesicle within the acidic vacuolar compartment have not yet been completed. This observation highlights that the pH sensing capabilities of Rosella can be used to monitor membrane continuity or integrity, although we do not have the optical resolution in these experiments to observe the ultrastructural organisation of the membranes themselves.

Mutant yeast cells lacking specific vacuolar enzyme activities required for efficient disassembly of membranes delivered by autophagy (e.g., *atg15Δ*) when starved of nitrogen accumulate a large number of Rosella labelled vesicles **(Fig. 4C)**. Some of these vesicles are both red and green indicating high pH whilst others appear only red indicating that they have a low pH-internal environment typical of the vacuole lumen. These results indicate that autophagic vesicles delivered into the lumen can retain their membrane integrity within the milieu of the resident hydrolases in the absence of the *ATG15* gene product, a putative lipase (Epple et al., 2001).

2.3.2 Monitoring autophagy in mammalian cells using Rosella.

The yeast vacuole is a relatively large and readily recognisable organelle, often accounting for much of the cell volume **(Fig. 4)**. Monitoring delivery of fluorescent cargo to the vacuole therefore is relatively simple. In contrast, the internal membrane structure of the mammalian cell is considerably more intricate and constitutes a profusion of vesicular compartments of various sizes. The mammalian lysosome is a much smaller organelle compared to the vacuole, usually present in large numbers that are distributed throughout the cytosol. The task of visualising delivery of cellular material to the lysosome is accordingly more complex and often requires specific labelling of the acidic organelle with proprietary dyes such as Lysotracker or Lysosensor (Klionsky et al., 2007b). The pH–sensing capability of Rosella allows the delivery of labelled material to be followed without the use

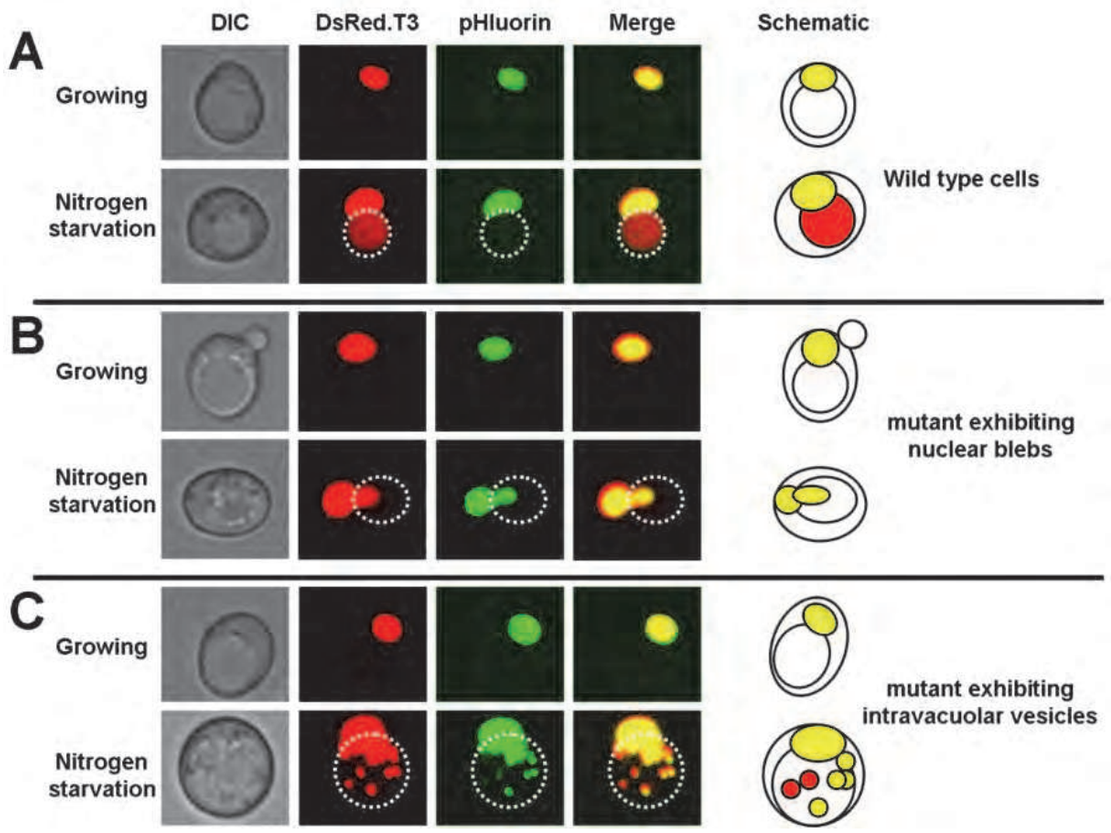

Fig. 4. n-Rosella in yeast: (A) Wild type cells expressing n-Rosella were imaged using fluorescence microscopy under growing and nitrogen starvation conditions. Accumulation of diffused red fluorescence in the vacuole after 24 h of commencement of nitrogen starvation indicates nucleophagy. (B) The absence of expression a particular gene product essential for nucleophagy influences nuclear morphology and abrogates correct delivery of n-Rosella to the vacuole. Nuclear blebs remain red and green indicating high pH environment. (C) The absence of the *ATG15* gene abolishes degradation of n-Rosella derived vesicles in the vacuole. Some intravacuolar vesicles are both red and green (indicating high pH environment) whereas others are only red (indicating low pH environment). The schematic (right) represents an interpretation of the image data. White dashed circles highlight the limits of the vacuole.

of additional probes to highlight the location of the lysosome. We next demonstrate in mammalian cells that Rosella can be used to monitor delivery of the cytosol or mitochondrion to the lysosome.

HeLa cells maintained in a replete growth medium and transfected with an expression vector encoding c-Rosella (Rosella without any additional targeting sequence) **(Fig. 3B)** when imaged using fluorescence microscopy showed both strong red and green fluorescence distributed throughout the cytosol. Rosella appears to have restricted access to the nuclear compartment (less intense staining) and is completely excluded from other compartments **(Fig. 5)**. Importantly, only 1-2 red and weakly green puncta/cell were observed **(Fig. 5A,** white arrows) suggesting that Rosella has accumulated in a relatively acidic compartment such as a lysosome. These puncta correspond to autophagolysosomes,

and represent fusion of an autophagosome carrying the Rosella cargo and a lysosome. Low numbers of puncta observed under growth conditions are consistent with basal autophagic activity and the homeostatic role of autophagy under these conditions.

Rapamaycin, an inhibitor of mTor (mammalian Tor), has been used in numerous studies to induce autophagy in HeLa cells (Ravikumar, et al., 2006). Following 4 h incubation in the presence of rapamycin (0.2µg/ml) a ~10-fold increase in the number of strongly red fluorescent puncta that were only weakly green fluorescent and corresponding to autophagolysosomes was observed **(Fig. 5A**, white arrows**)**.

The lysosome can be independently labelled using acidotropic dyes that accumulate in the lumen of the organelle (Klionsky et al., 2007b). The blue fluorescence emission of LysoTrackerBlue-White (LTBW) in the lysosome can be imaged together with the red and green emission of Rosella. In a separate experiment, prior to treatment with rapamycin to induce autophagy, lysosomes in Rosella-transfected HeLa cells were labelled with LTBW

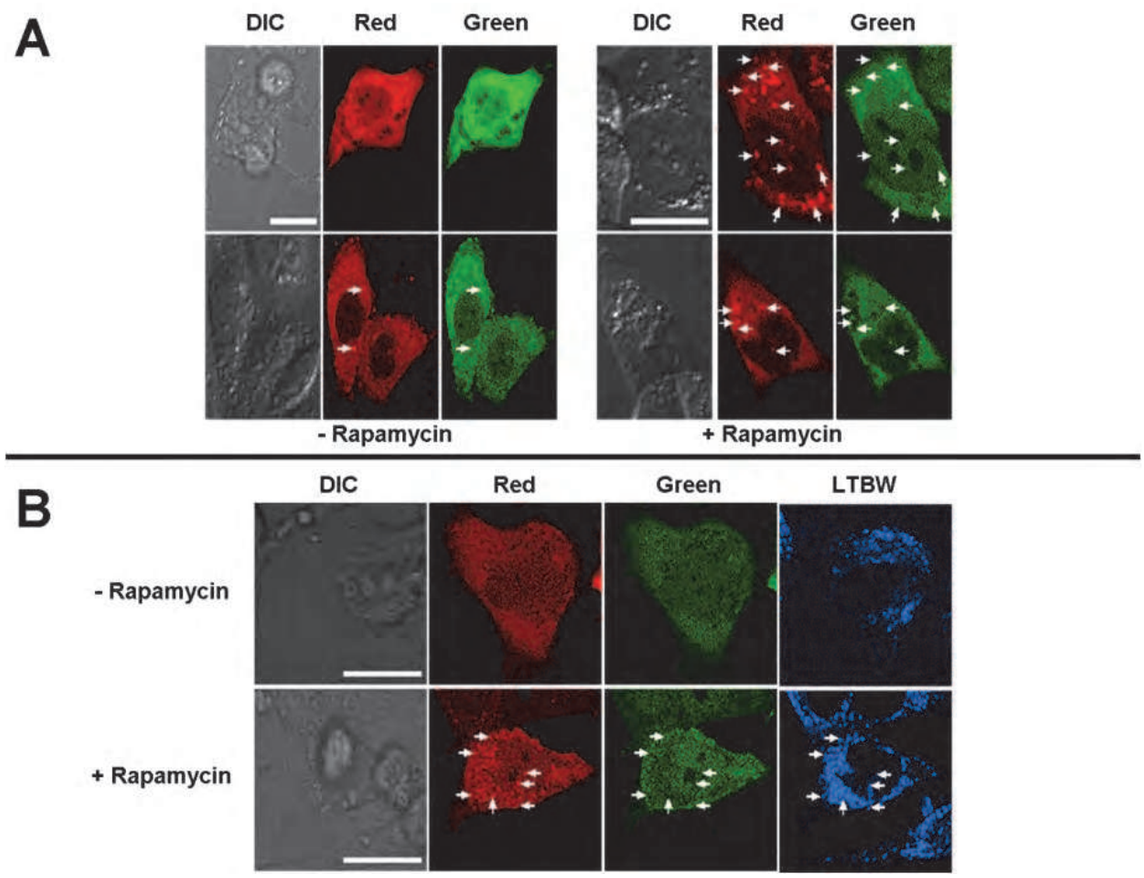

Fig. 5. Rosella can monitor autophagy in HeLa cells.

(A) HeLa cells expressing c-Rosella were imaged 24 h post-transfection for red and green fluorescence (left panel). 1-2 red puncta (white arrows) lacking green fluorescence and corresponding to uptake of cytosolic Rosella are visible in each cell. The number of red puncta lacking green fluorescence increased after incubation in the presence of rapamycin (0.2µg/ml) for 4 h (right panel). (B) In a separate experiment cells were incubated with LysoTrackerBlue-White (LTBW) to label lysosomal compartments. The scale bar is 20 µm.

(Fig. 5B). The puncta were both red and blue fluorescent, but not green fluorescent suggesting that these vesicles represent lysosomal derived compartments.
We next investigated whether Rosella was suitable for monitoring mitophagy in HeLa cells. For these experiments mt-Rosella (a variant of Rosella fused at its N-terminus to the mitochondrial targeting sequence of subunit VIII of cytochrome *c* oxidase; **Fig. 3B)** was expressed in HeLa cells grown in replete growth medium and visualised by fluorescence microscopy. Images of individual live cells show both bright red and green fluorescence restricted to a filamentous network distributed throughout the cell, consistent with a mitochondrial location **(Figs. 6A & 6B)**.

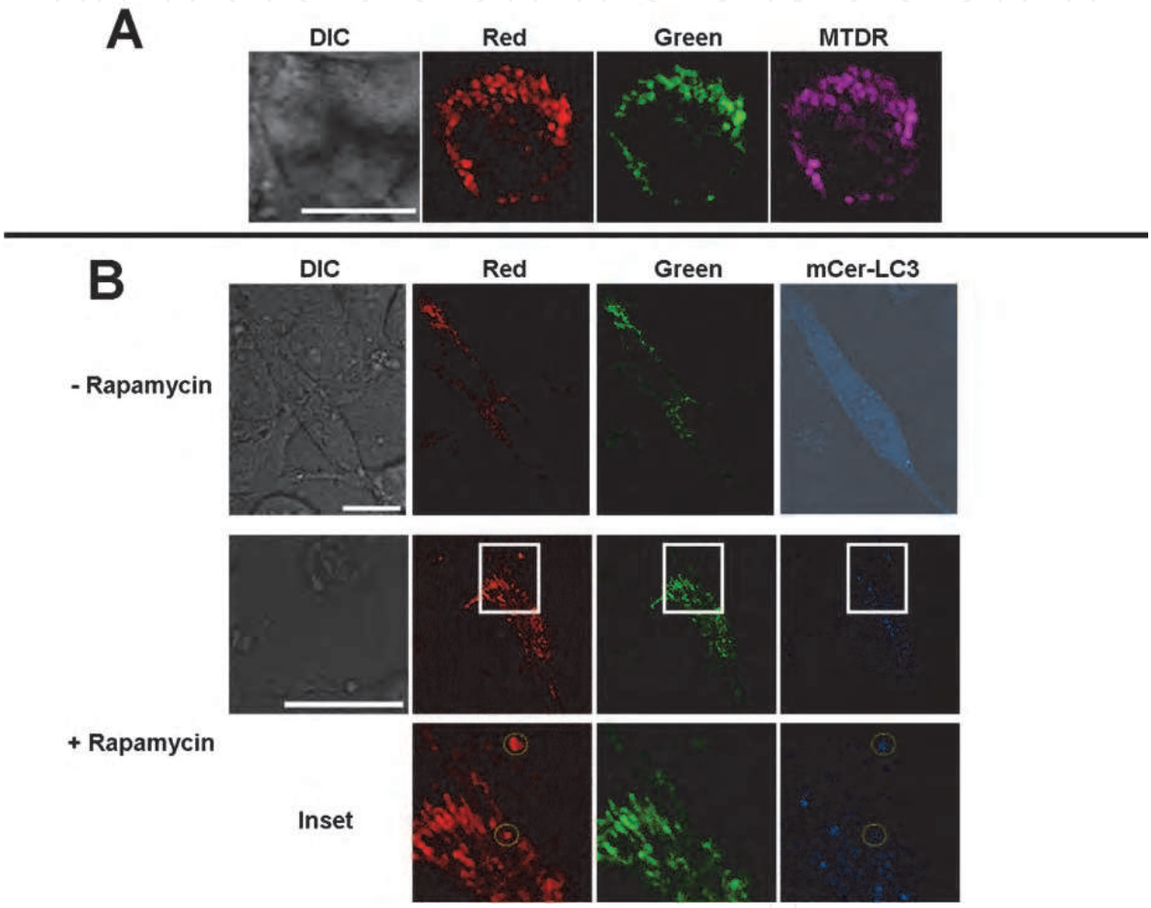

Fig. 6. Rosella can be used to monitor mitophagy in mammalian cells.
(A) DIC and fluorescence images are shown for HeLa cells transfected with an expression vector encoding m-Rosella. Cells were labelled after transfection with a far-red fluorescent mitochondrial probe, MitoTracker Deep Red (MTDR), whose emission is distinct from those of Rosella. (B) HeLa cells were co-transfected with expression vectors encoding m-Rosella and mCer-LC3, and subsequently incubated for 12 h in growth medium containing 0.2µg/ml rapamycin (+ Rapamycin) to induce autophagy. Control cells were not treated with the inducer (-Rapamycin). The white outlined inset region is shown enlarged. Yellow circles highlight red vesicles that co-localise with mCer-LC3, but contain little or no green fluorescence emission. The scale bar is 20 µm.

To confirm efficient targeting of mt-Rosella to the mitochondrion, transfected cells were incubated with the far-red fluorescent mitochondrial probe MitoTracker Deep Red (MTDR) **(Fig. 6A)** (Hallap et al., 2005). The far-red fluorescence emission of MTDR was observed to co-localise with the red and green fluorescence of mt-Rosella. Collectively, these results show that Rosella is efficiently imported into mitochondria, and subsequently becomes both red and green fluorescent.

Next, HeLa cells were co-transfected with expression vectors encoding mt-Rosella or a cyan FP (mCer) fused to the N-terminus of LC3. mCer-LC3 labels the autophagosome for reasons indicated in Figure 1. Transfected cells were cultured for 12 h without (control) or with the addition of rapamycin (0.2μg/ml) and imaged by fluorescence microscopy **(Fig. 6B)**. In control cells not stimulated with rapamycin, the presence of 1-2 cyan puncta per cell indicates autophagy occurring at a low homeostatic level. Since LC3-II will label autophagosomes resulting from both non-selective and selective autophagy, both of which will be induced by rapamycin, it is not expected that the puncta would exhibit the red fluorescence of mt-Rosella. Images of cells stimulated with rapamycin showed the presence of numerous cyan puncta indicating the recruitment of the LC3-II to the autophagosome **(Fig. 6B)**. Selected regions of the image (inset) are enlarged to highlight several autophagosomes that co-localise with bright red fluorescence, and therefore contain mitochondrial material labelled with Rosella. Green fluorescence emission is very weak or non-existent indicating that the pH inside the vesicles is relatively low and suggests that these autophagosomes have fused with lysosomes to form autophagolysosomes. Collectively, these data indicate that mt-Rosella can be used to monitor the delivery of mitochondrial contents to the lysosome.

3. Conclusions and alternative approaches

A better understanding of the molecular mechanism of autophagy in living cells and tissues is essential for the development of new therapeutic strategies to treat disease (Fleming et al., 2011). Accordingly, there is a need for the validation of reliable, meaningful and quantitative assays to monitor autophagy in live cells (Klionsky et al., 2007b; Klionsky et al., 2008; Mizushima et al., 2010).

Increased interest in selective forms of autophagy highlights the need to develop biosensors suitable for monitoring autophagy of specific targets. Exploiting components of the molecular mechanism such as LC3 to follow autophagy have proven to be particularly useful strategy, and LC3 tagged with a fluorescent protein remains the most commonly used marker of the autophagosome. However, such approaches involve additional labelling to identify target material. Labelling the target with Rosella allows delivery of the material to the acidic vacuole/lysosome to be followed by exploiting the unique pH-sensitive dual emission properties. Nevertheless, scope remains to improve development of new selective probes.

Biosensors suitable for high throughput, high content applications such as large scale drug or genetic screens are required. Although in some experimental regimes (e.g., yeast nucleophagy) the dual emission output Rosella can be analysed using conventional FACS analysis, sensitivity is somewhat reduced as the spatial information is lost and the assay relies on integrating the total red and green fluorescence emission from each cell (Rosado et al., 2008). New instrument technology such as imaging flow cytometry, an example of which is manufactured by the Amnis Corporation (https://www.amnis.com/autophagy.html),

would provide access to both spatial and colour information in cell populations (Lee et al., 2007). Our preliminary experiments in yeast cells suggest that this approach has potential but requires further validation and improvements under both physiological and autophagy-induced conditions (Rosado et al., 2008).

The development of biosensors with considerably improved signal-to-noise ratio may be possible using alternative probe technologies based on fragment complementation. Fragment complementation for a variety of different fluorescent proteins is now available (Kerpolla, 2006). The technology might be implemented to measure autophagy in one of several ways. For example, yeast cells in which one FP fragment is targeted to the mitochondrion and the complementing fragment targeted to the vacuole might be expected to have strongly fluorescent vacuoles *only* when mitophagy has occurred. Delivery of mitochondrial material including the FP fragment to the vacuole would allow re-constitution of a functional FP by fragment complementation. Cells would be otherwise non-fluorescent providing for a high signal-to-noise ratio. A similar and considerably more sensitive biosensor might be developed along similar lines if the FP is substituted for a member of the light-emitting luciferase family (Villalobos et al., 2010). Finally, it may be possible for an inactive pro-enzyme such as acid protease to be used to label targets. The enzyme would be proteolytically activated in the acidic lumen of the vacuole which would then be detected by incubation of cells with a cell permeant quenched fluorescent peptide substrate.

Given the interest in autophagy, it is likely in the near future that some of these ideas will result in the development of new sensitive and selective probes for this process.

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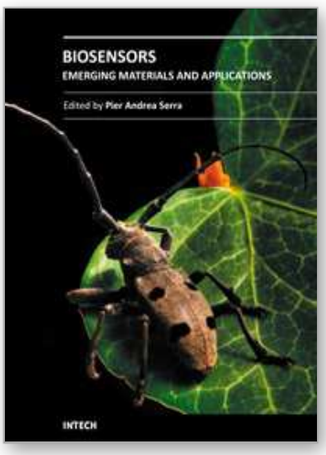

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A biosensor is a detecting device that combines a transducer with a biologically sensitive and selective component. Biosensors can measure compounds present in the environment, chemical processes, food and human body at low cost if compared with traditional analytical techniques. This book covers a wide range of aspects and issues related to biosensor technology, bringing together researchers from 19 different countries. The book consists of 27 chapters written by 106 authors and divided in three sections: Biosensors Technology and Materials, Biosensors for Health and Biosensors for Environment and Biosecurity.

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