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The Use of DQ-BSA to Monitor the Turnover of Autophagy-Associated Cargo

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Abstract

There is increasing evidence documenting the critical role played by autophagic and autophagy-associated processes in maintaining cell homeostasis and overall systemic health. Autophagy is considered a degradative as well as a recycling pathway that relies on encapsulated intracellular components trafficking to and fusing with degradative compartments, including lysosomes. In this chapter, we describe the use of DQ™-BSA to study autophagosome–lysosome fusion as well as a means by which to analyze hybrid autophagic pathways. Such noncanonical pathways include LC3-associated phagocytosis, better known as LAP. Both autophagosomes and LAPosomes (LC3-associated phagosomes) deliver cargo for degradation. The use of fluorescent DQ™-BSA in conjugation with autophagic makers and biomarkers of hybrid autophagy offers a reliable technique to monitor the formation of autolysosomes and LAPo-lysosomes in both fixed- and live-cell studies. This technique relies on cleavage of the self-quenched DQ™ Green- or DQ™ Red BSA protease substrates in an acidic compartment to generate a highly fluorescent product.

1. INTRODUCTION

The cell turns over its own constituents in a regulated manner to maintain homeostasis, in a process called autophagy, derived from the Greek word for “self-eating.” Numerous components of the autophagic pathway are multifunctional, forming a nexus of cross talk with other cellular processes to help define cell type-specific functions of autophagy and processes associated with autophagy-related proteins. Such processes include metabolism, hybrid phagocytosis, membrane transport, and host defense strategies.

Degradation of both intracellular and extracellular material is a crucial function of phagocytic cells. Two seemingly independent engulfment pathways, phagocytosis and autophagy, deliver material to lysosomes for degradation. Macroautophagy, hereafter referred to as autophagy, is the major catabolic pathway required for the lysosome/vacuolar degradation of cytoplasmic proteins and organelles. Through canonical autophagy, intracellular substrates are enwrapped as cargo by double membrane structures known as autophagosomes; thus allowing for bulk turnover of cytoplasmic components, enabling among other functions, the survival of nutrient-deprived cells. Upon nutrient deprivation, a serine-threonine kinase, UNC-51-like kinase (ULK), is released from its mammalian target of rapamycin (mTOR)-mediated inhibition (Jung et al., 2009; Kim, Kundu, Viollet, & Guan,

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2011); in concert with the class III phosphatidylinositol-3 kinase, Vps-34 complexed with Beclin1, it recruits and activates components of the Atg5/12/16L conjugation system (Funderburk, Wang, & Yue, 2010). The Atg5/12/16L multimeric complex regulates LC3 lipidation by phosphatidylethanolamine to form lipidated LC3, called LC3II. LC3II is necessary for autophagosome elongation and closure in cargo engulfment. LC3-containing autophagosomes subsequently fuse with lysosomes in an apparent LC3II-dependent manner to facilitate degradation of intracellular components (Esclatine, Chaumorcel, & Codogno, 2009; Jahreiss, Menzies, & Rubinsztein, 2008). Details of autophagy-associated proteins and their specific functions are reviewed in Klionsky et al. (2016).

Not all LC3-containing intracellular vesicles are autophagosomes; phagosomes can be targeted by autophagy proteins in an autophagosome-independent manner. In some epithelial cells and macrophages, phagocytosis activates the Vps34/beclin1 and Atg5/12/16L conjugation systems resulting in lipidation of LC3 directly onto the single membrane (nascent) phagosome (Florey, Kim, Sandoval, Haynes, & Overholtzer, 2011; Florey & Overholtzer, 2012). In this hybrid pathway, commonly known as LC3-associated phagocytosis (LAP), the LC3II-decorated phagosome fuses with lysosomes for degradation. Autophagosome-independent, LC3-associated degradative events exhibit common themes that define the process of LAP: LC3 recruitment to phagosomes occurs under nutrient replete conditions in which mTOR is active with canonical autophagy inhibited. LAP is however dependent on Vps34/beclin1 and Atg5/12/16L. It is becoming increasingly evident that LAP processes are a means by which phagocytes monitor their contents to ensure complete degradation of ingested materials. LAP requiring processes include phagocytosis of dead cells (Martinez et al., 2011), the degradation of photoreceptors, and recycling of visual pigments (Frost et al., 2015; Frost, Mitchell, & Boesze-Battaglia, 2014; Kim et al., 2013) and pathogen degradation (Sanjuan et al., 2007). Most recently, LAP has been shown to inhibit the autoimmune response (Martinez et al., 2016).

Independent of whether LC3 decorates the inner and outer bilayers of double membrane autophagosomes or just the outside of ingested phagosomes, the ultimate fate of the contents of these structures is degradation upon fusion with lysosomes. Historically, the function of degradative compartments was often measured as proteolytic activity; highly substituted fluorescein conjugated derivatives of proteins served as biomarkers of a cell’s degradative efficiency and capacity. A limitation of such fluorescein derivatives is their pH sensitivity over physiological lysosomal pH ranges; they provide limited detection below pH 8.0. Newer fluorescent compounds have emerged including the BODIPY© family of probes which are insensitive to pH changes from pH 3 to 11. Here we provide a method by which the bovine serum albumin derivative DQ™-BSA is utilized to detect the formation of the autolysosomes and the quantitation of degradative cargo using confocal imaging.

2. MATERIALS

The DQ™BSA conjugate is a derivative of BSA that is labeled to such a high degree with either the green-fluorescent BODIPY® FL or the red-fluorescent BODIPY® TR-X dye that the fluorescence is self-quenched. Spectral properties of DQ™Green and DQ™Red BSA allow for extensive utility in a variety of applications. They both provide low background

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fluorescence and provide high signal-to-noise ratios upon digestion. The cleavage of DQ™ Green BSA results in the release of fragments which have λ_{ex} = 505 nm and λ_{em} = 515 nm. In the case of DQ™ Red BSA the proteolytic fragments have λ_{ex} = 590 nm and λ_{em} = 620 nm. As the DQ™-BSA enters an acidic environment proteases cleave the previously quenched polypeptide to generate fluorescent peptide fragments as illustrated schematically in Fig. 1.

DQ-BSA probes are available as a lyophilized powder that if protected from light are stable for up to 6 months at −20°C. After reconstitution, the solution is stable for 3–4 weeks at 4°C (protected from light; wrap vial in foil). We routinely reconstitute with sterile PBS.

The methods below describe the use of DQ™ Green BSA in the visualization and semiquantitation of autolysosome formation as well as the delivery of LC3-associated cargo to lysosomes (Frost et al., 2015). Ha et al. (2010), utilized DQ™ Red BSA to measure the role of autophagic flux in Anthrax Toxin lethal factor delivery using confocal imaging and FACS. Both approaches effectively measure the convergence between an autophagic or autophagocytic compartment and a functional lysosome; in the case of ingested phosphatidylserine positive photoreceptor outer segments (POS), the autophagy-associated process is LAP (LC3-phagolysosomes) while for anthrax toxin it is autolysosome formation. Here we describe DQ™-BSA's use for autolysosomes as well as a modification in which we measure LAP.

The individual steps for monitoring autolysosome and LC3-phagolysosome formation are described later. This protocol can be simplified for nonpolarized cells by ignoring steps in Section 3.

3. ESTABLISHMENT OF POLARIZED EPITHELIAL CELL CULTURES

1. ARPE19 cells (ATCC CRL-2302) or human fetal RPE (hfRPE) cells are seeded on 12-well Transwell filters with 0.4 μ*M* pore size (Corning 3460, Oneonta, NY) at an initial seeding density of 1.6 × 10^5 cells/well in the apical chamber. Routinely, 0.5 and 1.5 mL of DMEM/F12 (1:1) (Gibco 11330032) with 10% FBS is added to the apical and basal chambers, for ARPE19 cells, while hfRPE cells utilized Advanced MEM (Gibco 12492013) with 1% pen/strep (Gibco 15140122, Grand Island, NY), 1% glutamine (Corning 250054), 125 mg taurine (Sigma T0625), hydrocortisone (Tocris Bioscience 4093), 0.0065 μg triiodo-thyronin (Sigma T5516) with 5% FBS. Cells are subsequently incubated at 37°C and 5% CO_2. Frost et al. (2015) and Adijanto et al. (2014) provide detailed protocols for ARPE19 and hfRPE cultures, respectively.

2. After initial plating, the formulation of the media is changed to 1% FBS, and cells are fed twice a week.

3. To confirm barrier function, the transepithelial resistance (TER) of mature cells is measured using an Epithelial Volt–Ohm Meter (EVOM2). Upon cell maturity, usually within 4 weeks, the TER is routinely ~50 Ω cm^2 for ARPE19 cells and 500 Ω cm^2 for hfRPE.

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4. INCORPORATION OF DQ™-BSA CONJUGATES

1. Cells at 65–85% confluence are incubated with 10 μg/mL DQ™-BSA in complete media at 37°C for 30–60 min. When using polarized cells, DQ™-BSA is added to the apical chamber and incubated at 37°C for 1 h. DQ™Green BSA (D12050) and DQ™Red BSA (12051) are both obtained from Molecular Probes.
2. Excess probe is removed by washing cells three times with PBS containing 0.5 m*M* $MgCl_2$ and 0.9 m*M* $CaCl_2$ (PBS-CM, Thermo-Fisher, Cat# 14040133).
3. Autolysosome formation can be monitored by confocal imaging as indicated later.

5. MONITORING AUTOLYSOSOME FORMATION

Monitoring of autolysosome formation requires that the DQ™-BSA traffic to lysosomes where acidic proteases generate a highly fluorescent product. Autolysosomes are defined herein as intracellular structures in which there is colocalization of cleaved DQ™-BSA fragments (a marker of lysosomes) with fluorescently tagged-LC3 or endogenous LC3 (a marker of autophagic structures).

1. To identify autophagosomes or LAPosomes, fluorescently labeled LC3 can be expressed using transient transfection techniques. In addition, numerous cells lines have been developed that stably express GFP-LC3 or mCHERRY-LC3. In addition, several mouse models express eGFP-LC3 or mCHERRY-LC3 (Iwai-Kanai et al., 2008). GFP-LC3 mice are available from RIKEN BioResource Center (RBRC No. 00806, Japan), and mCHERRY-LC3 mice are available from Jackson Laboratories (Stock No. 021853). In addition, a tandem tagged LC3 mouse is available from Jackson Laboratories (Stock No. 027139) (Li, Wang, Hill, & Lin, 2014). Endogenous LC3 is labeled as described in more detail later (Section 7.1).
2. When monitoring autolysosomes using transiently transfected, fluorescently tagged LC3, transfections should be performed 24–36 h prior to DQ™-BSA addition.
3. Autophagosome formation can be stimulated using several different experimental protocols depending on the nature of the studies proposed; we will describe pharmacologic stimulation with rapamycin and serum starvation.

5.1 Rapamycin Stimulation

Rapamycin acts to remove the mTOR-mediated inhibition of the Ulk1 complex thereby resulting in an increase in autophagy. Cells are incubated with 100 n*M* rapamycin (Enzo Life Sciences, BML-A275-0005) for 4 or 24 h and subsequently incubated with DQ™-BSA, follow steps 1–4 in Section 5.3.

1. Rapamycin activity is confirmed as loss of phospho-S6 by immunoblot using anti-phosphoS6 (Cell Signaling, 2211s).

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5.2 Serum Starvation

To remove media wash cells three times in PBS. To serum starve change to 0.25% FBS. To control cells, add complete culture media DMEM/F12 with 1% fetal bovine serum (Sigma, 12003C) and 5% Penicillin–Streptomycin (Sigma, P4333). Incubate cells at 37°C for 4 h.

5.3 Evaluation of Autolysosome Formation

1. Incubate control, rapamycin treated or serum-starved cells with DQ™-BSA as described earlier (Section 4) and wash three times with PBS.
2. For confocal image analysis, plate cells on coverslips prior to treatment and fix in 4% paraformaldehyde (PFA) after treatment.
3. The fluorescence degradation products of DQ™-BSA are imaged using standard techniques. A representative example of a multifluor confocal image is shown in Fig. 2A. In this example, imaging was performed on a Nikon A1R confocal microscope and images analyzed using Nikon Elements software 4.1. In codistribution analyses with LC3, cells are imaged and codistribution analyzed using a binary submask Pearson's coefficient cut-off of at least >0.55. The extent of autolysosome formation is indicated by DQ™-BSA colocalized with RFP-LC3 as shown for a 2-h incubation in Fig. 2B.
4. Alternatively, serum-starved cells are analyzed using live-cell confocal imaging. In this way, the uptake and localization of DQ™-BSA is measured in real-time in combination with fluorescent-LC3. The extent of RFP-LC3-DQ™-BSA uptake observed by live-cell confocal imaging, beginning at 2 min is depicted in Fig. 2C.

6. MONITORING LC3-ASSOCIATED PHAGOLYSOSOME FORMATION

The generation of highly fluorescent proteolytic products in lysosomes allows us to monitor the delivery of autophagy-dependent cargo to these organelles. In the example below, we provide a semiquantitative method by which to analyze a hybrid phagocytosis–autophagy-dependent process; LC3-associated phagocytosis known as LAP. Here LC3-phagolysosomes are defined as intracellular structures in which there is a triple colocalization between cleaved DQ™-BSA fragments, fluorescently tagged-LC3 or endogenous LC3 and fluorescently labeled phagosomes (Frost et al., 2015).

1. Incubate polarized RPE cells with 10 μg/mL DQ™-BSA in the apical chamber for 1 h at 37°C.
2. Wash three times in media prior to the addition of fluorescently labeled outer segments or other phagocytosed material, i.e., latex beads, zymogens, etc. In this example, we add purified POS, the endogenous particles phagocytosed by the RPE.
3. Add Alexa Fluor 647-labeled POS (AF647-POS) at a density of ~10 particles per cell directly to the media on the apical side for 2 h at 37°C.

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4. After 2 h quench the unincorporated AF647-POS with 0.2% Trypan Blue (Corning, 25-900-cl) for 10 min at 37°C.
5. Wash three times in PBS-CM.
6. Fix inserts in 4% PFA, wash in PBS, and mount or process for immunofluorescence staining.

7. IMMUNOFLUORESCENCE ANALYSIS

7.1 Labeling Endogenous LC3

1. Permeabealize inserts with ice-cold methanol at −20°C for 10 min.
2. Wash with PBS and incubate in PBS for 30 min to rehydrate.
3. Incubate inserts in 4% BSA (Sigma A7284) in PBS containing 0.05% Triton TX-100 (PBST) for 60 min at 37°C to block prior to labeling with appropriate antibodies.
4. After blocking, incubate inserts at 37°C for 60 min with anti-LC3 rabbit polyclonal antibody 1:150 (Cell Signaling, Catalog no. 2775s).
5. Wash inserts three times in PBST.
6. Incubate with secondary antibody, Alexa Fluor 594 donkey anti-rabbit IgG (Invitrogen R37119) at 1:1000 and Hoechst 33258 (Thermo-Fisher, H3569) at 1:10,000 (37°C for 60 min) and wash three times in PBS.

7.2 Confocal Imaging

1. In preparation for microscopy, cut the filters from the inserts and mount in Cytoseal (Electron Microscopy Sciences, 18006).
2. For polarized cell analysis, capture *Z*-stack images of 1 μm apical to basolateral sections on a Nikon A1R laser scanning confocal or comparable microscope with a 60× water objective (NA 1.2).
3. A representative example of the triple colocalization profiles of Texas-Red (TR)-OS, LC3, and DQ™Green BSA is shown in Fig. 3A, with subsequent quantitation of OS association with DQ™Green BSA-LC3 positive structures in Fig. 3B.

7.3 Important Considerations in Experimental Design

1. The internalization and transport of DQ™-BSA to the lysosomes is compromised in the presence of PI3K inhibitors, such as wortmanin and upon the overexpression of motor proteins and Rabs (dominant negative mutants) as well as upon alterations in phospholipid phosphatases, including SH-2-containing inositol 5′-polyphosphatase 1 (SHIP1) and phosphatase and tensin homolog (PTEN). To avoid inefficient localization of DQ™-BSA, cells should be incubated overnight with DQ™-BSA prior to pharmacologic manipulation. However, if DQ-BSA is used as an indicator of endolysosomal trafficking the

levels of protein(s) or toxin of interest should be altered prior to DQ™-BSA. A detailed description of such as approach is provided (Corrotte, Fernandes, Tam, & Andrews, 2012).

2. Treatment with inhibitors, including chloroquine or bafilomycin A, a v-ATPase inhibitor, that block endosomal acidification or autophagosome–lysosome fusion led to a strong reduction in DQ-BSA fluorescence confirming that cleavage of this dye requires residence in acidic compartments (Corrotte et al., 2012; Goeritzer et al., 2015).

3. A caveat to interpretation of results with DQ™-BSA is that this probe may be dequenched in late endosomes (Authier, Posner, & Bergeron, 1996; Goebeler, Poeter, Zeuschner, Gerke, & Rescher, 2008). Given the dynamic nature of the late-endosome to lysosome conversion, we recommend several complementary approaches that utilize both functional convergence and organelle biomarker identification, including cathepsin D, Lysotracker, or LAMP1 (Frost et al., 2015). Complementary LC3-DQ™-BSA codistribution analysis can be performed using FACS (Ha et al., 2010).

8. SUMMARY

We describe basic protocols to assay the last step of autophagy, one shared with LAP, the fusion of an LC3 positive compartment with a degradative lysosome. Incorporation of the fluorogenic DQ™Green or Red BSA probe is an easy and fast method by which to monitor starvation or pharmacologically induced autophagy in fixed or live cells. Although the colocalization of phagocytosed particles with LC3 assesses the formation of a LAPosome, it does not address whether that structure then appropriately traffics to a lysosome for degradation. Therefore, detecting the colocalization of LC3-associated cargo with DQ™-BSA is the one of the most reliable methods for monitoring fusion between a LAPosome and an active proteolytic compartment, either late-endosome or lysosome. This method is in many ways superior to colocalization studies that rely only on the presence of lysosomal enzyme. Typically, such immunofluorescence studies do not assess the compartment's function, as the presence of a protease, for example, cathepsin D does not necessarily confirm that the compartment is functional. The use of DQ™-BSA circumvents this problem since fluorescence is only observed in a proteolytically active compartment.

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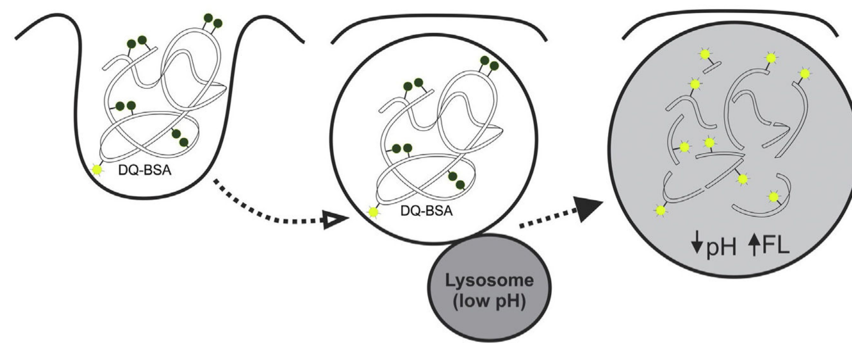

Fig. 1.

Schematic representation of self-quenched DQ™ Green BSA upon cleavage by proteases in acidic compartments. Proteolysis results in fragment formation with fluorescence dequenching that is observed as an increase in fluorescence intensity.

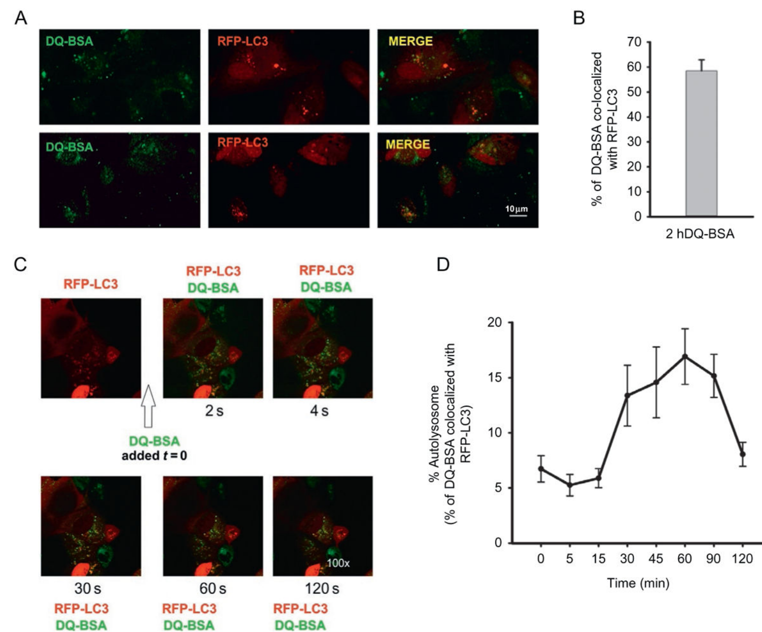

Fig. 2.

Serum-starved ARPE19 cells accumulate autolysosomes. (A) RFP-LC3 transfected cells were serum starved for 4 h in 0.25% FBS. Starved cells were incubated with DQ™ Green BSA. Autolysosomes were identified as LC3-positive (*red*)—green DQ-containing structures, with a Pearson's coefficient cut-off of 0.55. (B) Percentage of total lysosomes representing autolysosomes is shown at 2 h after rapamycin treatment (100 n*M*). (C) Live-cell confocal imaging, serum-starved (for 4 h) RFP-LC3-transfected ARPE19 cells grown on coverslips were placed in a temperature-controlled stage and loaded with DQ™ Green BSA. The uptake of DQ™ Green BSA and subsequent fusion with RFP-LC3 autophagosome was recorded using time-lapse microscopy. Images were captured every 2 s, images represent DQ-BSA and RFP-LC3, at the indicated time points. (D) In another series of experiments, the extent of colocalization between DQ-BSA and RFP-LC3 was determined exactly as described in (C) except cells were serum starved for 1 h. The results are mean ± SEM of five cells in each of three individual live-cell assays, with a Pearson's coefficient cut-off of 0.55.

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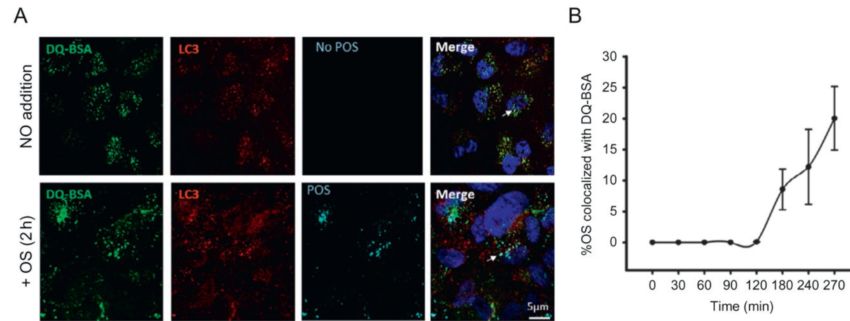

Fig. 3.

LC3-OS-lysosome association. (A) DQ™Green BSA containing ARPE19 cells were fed Alexa Fluor 647-labeled POS at a density of 10 particles per cell directly to the media in the apical chamber for 2 h at 37°C. Phagocytosis was terminated and extracellular fluorescence quenched. Cells were fixed and stained for endogenous LC3. A representative image of each channel is shown and triple colocalization indicated with *arrows*. The average size of the particles quantified as LAPo-lysosomes was 0.65–0.89 μm. Scale bar is 5 μm. (B) Live-cell confocal imaging, ARPE19 cells grown on coverslips were loaded with DQ™ Green BSA. The coverslips, placed in a temperature-controlled stage, were fed Alexa Fluor 647-labeled POS and images recorded every 2 s for 4.5 h. The extent of colocalization was determined at each indicated time point 422 with Pearson's coefficient >0.55.