PROCEEDINGS OF SPIE

SPIEDigitalLibrary.org/conference-proceedings-of-spie

Fluorescence lifetime imaging of microviscosity changes during ER autophagy in live cells

Ying He, Soham Samanta, Wanjun Gong, Wufan Liu, Wenhui Pan, et al.

Ying He, Soham Samanta, Wanjun Gong, Wufan Liu, Wenhui Pan, Zhigang Yang, Junle Qu II, "Fluorescence lifetime imaging of microviscosity changes during ER autophagy in live cells," Proc. SPIE 10495, Biophotonics and Immune Responses XIII, 104950R (19 February 2018); doi: 10.1117/12.2293440

SPIE.

Event: SPIE BiOS, 2018, San Francisco, California, United States

Fluorescence lifetime imaging of microviscosity changes during ER autophagy in live cells

Ying He†, Soham Samanta†, Wanjun Gong, Wufan Liu, Wenhui Pan, Zhigang Yang*, Junle Qu*

Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province, College of Optoelectronic Engineering, Shenzhen University, Shenzhen, 518060, China.

*****Corresponding author: zhgyang@szu.edu.cn (ZYang); E-mail: jlqu@szu.edu.cn (JQu)

† These authors contributed equally to this work

Abstract. Unfolded or misfolded protein accumulation inside Endoplasmic Reticulum (ER) will cause ER stress and subsequently will activate cellular autophagy to release ER stress, which would ultimately result in microviscosity changes. However, even though, it is highly significant to gain a quantitative assessment of microviscosity changes during ER autophagy to study ER stress and autophagy behaviors related diseases, it has rarely been reported yet. In this work, we have reported a BODIPY based fluorescent molecular rotor that can covalently bind with vicinal dithiols containing nascent proteins in ER and hence can result in ER stress through the inhibition of the folding of nascent proteins. The change in local viscosity, caused by the release of the stress in cells through autophagy, was quantified by the probe using fluorescence lifetime imaging. This work basically demonstrates the possibility of introducing synthetic chemical probe as a promising tool to diagnose ER-viscosity-related diseases.

Keywords: Viscosity, ER-stress, Autophagy, FLIM, Molecular Rotor.

1 Introduction

Endoplasmic Reticulum (ER) is one of the important organelles in cells, which plays important roles in nascent protein manufacturing and post-processing. Abundant unfolded or misfolded proteins, accumulated in the ER could readily generate stress conditions,[1] which will then eventually activate corresponding cell response to release ER stress to restore the normal ER functions *via* two different pathways: ubiquitin-proteasome-dependent ERAD (type I) and autophagy lysosome dependent ERAD (type II), respectively.[1-3] In these biological processes, microenvironment, especially microviscosity in ER, will be changed with the varying health conditions or malfunction status of ER. Therefore, quantitative, real time monitoring of viscosity changes in ER can become a convenient method for diagnosing ER malfunctions related disease.

Biophotonics and Immune Responses XIII, edited by Wei R. Chen, Proc. of SPIE Vol. 10495, 104950R · © 2018 SPIE · CCC code: 1605-7422/18/$18 · doi: 10.1117/12.2293440

Generally, viscosity is known as one of significant environment factors, which predominantly governs various critical processes in biological system.[4] It plays essential roles in determining regular behaviors of protein fabrications such as protein interactions, mobility and folding in ER, which are largely dependent on local viscosity.[5,6] Abnormal changes of viscosity signifies the malfunction of ER which may lead to ER stress and even several diseases, such as atherosclerosis, diabetes, Alzheimer's disease, malfunctions of cell development and so on.[7-10] Hence, viscosity changes in ER can be used as an indicator to track the malfunction of ER. Recently, a new kind of fluorescent probes, termed as "molecular rotor" has been widely used to study the viscosity in micro-biological envoronments.[6,11-13] Essentially, the fluorescent 'rotors', capable of fluorescence lifetime imaging (FLIM) can be readily applied for quantifying biological viscosity in complex environment. In our previous work, couple of ratiometric fluorescent probes were developed to measure the exact viscosity inside cells *via* lifetime imaging.[6,11] However, in those reports, exact viscosity measurement around nascent proteins under different situations of ER, such as ER autophagy process, was not reconnoitered.

With above considerations, to monitor local viscosity changes in ER autophagy process, a multifunctional probe of BODIPY based fluorescent molecular rotor (**1**) was developed to study local viscosity by FLIM imaging. The probe can covalently bind to nascent VDPs in ER, which in turn inhibits the folding of the proteins and generates ER stress to facilitate the formation of the ER autophagosome to release the ER stress. The concurrent change in local viscosity has been quantitatively monitored during these processes *via* FLIM imaging using the probe **1** wherein the BODIPY played as a signaling fluorophore moiety and arsenate moiety is able to covalently attach to VDPs, which will in situ generate ER stress.

2 Materials and Methods

2.1 Materials

The HeLa cells were purchased from the American Type Culture Collection (ATCC) (VA, USA). All reagents for cell culture, ER-Tracker Red (BODIPY TR Glibenclamide), MitoTracker Deep Red FM, and LysoTracker Red DND-99 were purchased from Invitrogen (Oregon, USA). Methanol, DTT (Dithiothreitol), HFIP (1, 1, 1, 3, 3, 3-hexafluoro-2-propanol) and TCEP (Tris(2-carboxyethyl)phosphine hydrochloride) from Sigma–Aldrich were used as received without further purification. Amyloid β-Protein (1-42) was purchased from Bachem. Antibodies to Lamin B1 (H-90) and β-Actin (C4) were purchased from Santa Cruz Biotechnology (Santa Crus, US). Anti-VDAC 1 and VDAC 2 were obtained from Abcam (Cambridgw, UK). Sequencin-grade trypsin was from Promega (Wisconsin, US).

*2.2 Synthesis of the probe (**1**)*

The fluorescent probe (**1**) was synthesized following the procedure reported in our previously published work (Fig 1).[6,11] All the reactions were performed in oven-dried apparatus, and the solvent was freshly distilled. All the reagents were bought form commercial suppliers and used without further purification. A rotary evaporator was used to concentrate the reaction mixtures. Thin layer chromatography (TLC) was performed using glass-backed sheets of silica gel and visualized under a UV lamp (254nm and 365 nm). Column chromatography was performed to purify compounds by using silica gel 60 (200-400 mesh).

2.3 UV/Vis and fluorescence spectroscopy

Stock solutions of **1** (1 mM) was prepared in DMSO. Absorption spectrum was recorded using V-560 UV/VIS spectrophotometer (JASCO, Japan), and fluorescence spectra were recorded on

RF-5301 PC spectrofluorometer (Shimadzu) equipped with a xenon lamp. Other details including the excitation wavelength and the concentrations of experimental solutions are stated in the figure captions.

2.4 Formation of reduced BSA (rBSA) and oxidized BSA (oBSA)

rBSA was obtained by treating solution of BSA with 1 mM DTT overnight at 4 °C and oBSA was obtained by treating BSA with 100 μM H_2O_2 for 10 min at 25 °C. These samples were first diluted 50 times with distilled water and then the proteins were recovered by precipitation in 50% acetone for 2 h at -20 °C.

2.5 Cell culture

HeLa cells were cultured in DMEM medium, of which were supplemented with 10% (v/v) FBS (WelGene), penicillin (100 units/mL), and streptomycin (100 mg/mL) under 5% CO_2 and 95% humidity at 37 °C.

2.6 Confocal microscopy

24 hours before imaging, the cells were seeded on cover glass bottom dishes (SPL Life sciences Co., Ltd.) which was incubated in a humidified atmosphere containing 5% (v/v) CO_2 at 37 °C. Cell images were taken by a confocal laser scanning microscope (Leica SP8, Leica, Germany). Other information is available in the figure captions.

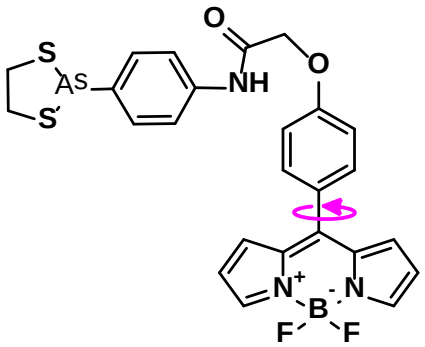
2.7 SDS-PAGE and fluorescence image of gels

The selectivity of **1** towards proteins or cells was verified by 10% SDS-PAGE. Samples were treated with **1** in de-ionized water (DW) or DMEM at 5% CO_2 and 95% humidity at 37°C for 1 h. After labeling, the samples were precipitated with 50% (v/v) acetone for 2 h at -20°C and then

mixed with SDS-PAGE loading buffer with tris (2-carboxyethyl) phosphine (TCEP) and the electrophoresis was started immediately. The gel was imaged by a fluorescent scanner (Typhoon FLA 9400, GE) upon 488 nm excitation with a band path filter ranging from 500 nm to 540 nm. The same gel was stained by coomassie brilliant blue (CBB) G250 after the fluorescent image was obtained. Other details including the excitation wavelength and emission filter are given in the figure captions.

3. Results and Discussions

To carry out the fluorescence imaging, the fluorescent probe (**1**) was synthesized first according to our previous work (Fig 1). The fluorescent probe (**1**) comprises two typical moieties in structure: (a) a rotating BODIPY fluorophore as viscosity sensitive unit and (b) a 1, 3, 2-dithiarsenolane containing group; to selectively label vicinal dithiols containing proteins. The key feature of designing the probe was to conjugate 1, 3, 2-dithiarsenolane moiety with the BODIPY moiety through a flexible chain, so that a molecular rotor behavior can be introduced in the system.



1

Fig. 1 Chemical structures of target molecular rotor (**1**)

To verify whether **1** is capable of showing adequate sensitivity towards the local viscosity changes or not, fluorescence responses of **1** towards arbitrary viscosity changes were studied in different ratios of water/glycerol mixtures. First, fluorescence spectra and fluorescence lifetime

decay of **1** (2 μM) were measured in binary solvents of water and glycerol (Fig. 2); which revealed that the fluorescence intensity of **1** at 516 nm enhanced significantly upon increasing the glycerol fraction (indicating increase in viscosity) at room temperature (25 °C) [Fig. 2(a)], when excited at 488 nm. It is noteworthy that the fluorescence intensity of **1** at 516 nm had increased linearly (R^2 = 0.99) up to 20 folds as the mixed solvents (water/glycerol) varied from water (*ca.* 1.00 cP) to 99% glycerol (*ca.* 950 cP) [Fig. 2(b)]. In the meanwhile, the fluorescence decay profiles of **1** were also measured on a time correlated single photon counting equipment (TCSPC) commercial DCS-120 system by changing the ratio of the mixed solvents from 100% water to 99% glycerol. As shown in Fig. 2(c), the fluorescence lifetime was increased with the enhancement of solution viscosity (higher glycerol content). It was noted that upon gradually enhancing the ratio of water/glycerol from 0 to 99% glycerol, the fluorescence lifetime of **1** (τ) had linearly (R^2 = 0.97) increased from *ca.* 0.2 ns to *ca.* 5.4 ns [Fig. 2(d)]. The linear relationship between the change in viscosity and the fluorescence intensity provided the scope of utilizing probe **1** for the quantification of local viscosity. It might be mentioned here that the free rotation around the C-C bond between BODIPY and the phenyl unit was gradually suppressed by increasing solvent viscosity, which resulted in the decrease of energy consumption in the form of non-radiations to show the observed fluorescent responses.

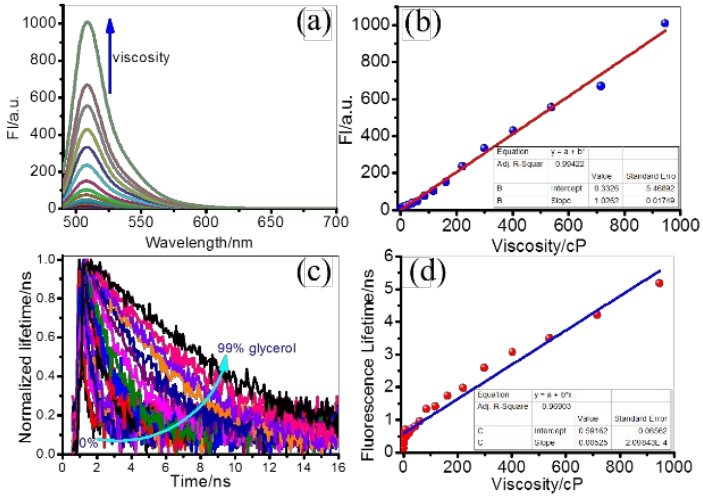


Fig. 2 Fluorescent spectral changes of probe **1** (2 μM) with the variations of solution viscosity in water-Glycerol (a), Linear relationship between fluorescence intensity and viscosity in mixed solvents (b), λ_{ex}=470 nm; Fluorescence decay of **1** in Water-Glycerol (c), and Linear relationship between fluorescence lifetime and solution viscosity in Water-Glycerol (d), respectively, λ_{ex}=470 nm and detected at ca. 515 nm, using a time correlated single photon counting equipment (TCSPC) commercial DCS-120 system.

The reactivity of **1** towards VDPs was checked with different proteins, wherein each protein was incubated with **1** to ascertain whether the probe **1** is covalently bound to the protein(s) or not. Bovine serum albumin (BSA) (oxidative form: oBSA) was first treated with dithiothreitol (DTT) to obtain reduced form of the protein (rBSA). Then rBSA was incubated with **1** (1 μM) at 37 ^{o}C through vortex mixing. As demonstrated in Fig. 3, the fluorescence intensity of **1** increased with the prolonging of incubation time [Fig. 3(a), 3(b)], and saturated at *ca.* 30 minutes, indicating towards the binding between **1** and VDPs. This can be further verified by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) experiments. As seen in Fig. 3(c), the reaction solution was used to perform gel electrophoresis. The protein bands, with molecular weight of *ca.* 66 KDa, became wider upon increasing rBSA concentration [Fig. 3(c)] as observed from both CCB staining and fluorescent imaging.

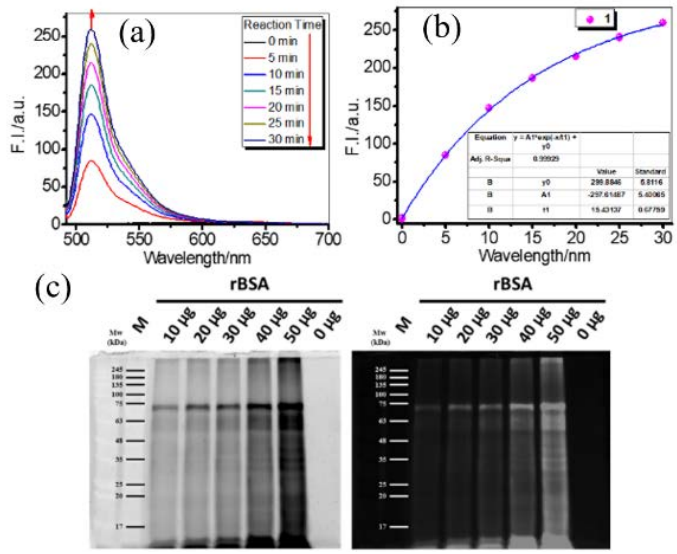


Fig. 3 Time dependent fluorescence spectra (a) and intensity (b) of **1** (1 μM) with Albumin (10 μM); after albumin (10 μM) incubated with DTT (0.1 mM) for 30 min, 37 ℃, added each of the probe (1 μM), excited at 488 nm, slit widths (3/3). SDS-PAGE experiments on the probe **1** covalently banding with different amount of reduced BSA [rBSA, Panel (c)].

Co-localization experiments were carried out not only to get an exclusive insight of the sub-organelle localization of **1** inside live HeLa cells but also to uncover the preferential orientation of **1** when it goes inside the live cells. Commercially available Mito-, Lyso- and ER-trackers were respectively incubated in the live HeLa cells for 5 minutes along with the probe **1** (1 μM). The confocal images were taken by a Leica SP8 microscope with separate excitation and collecting ranges. It can be seen from the merged images that **1** and ER-Tracker shows convincing yellow fluorescence in a punctuated pattern with the best overlapping fluorescence, revealing the major location of the probe **1** in the ER [Fig. 4(c)] with Pearson's coefficient: r = 0.99 and overlap coefficient: r = 0.99, respectively. However, Lyso- or Mito-trackers rendered only a partial co-localization with the probe **1** in a rarely visible manner [Fig. 4(f), 4(i)]. The Pearson's Coefficient and overlapping coefficient were calculated to be: r = 0.67 and r = 0.53 for

lyso- or mito-trackers respectively, which clearly substantiates that, the probe **1** can preferentially label the proteins in ER.

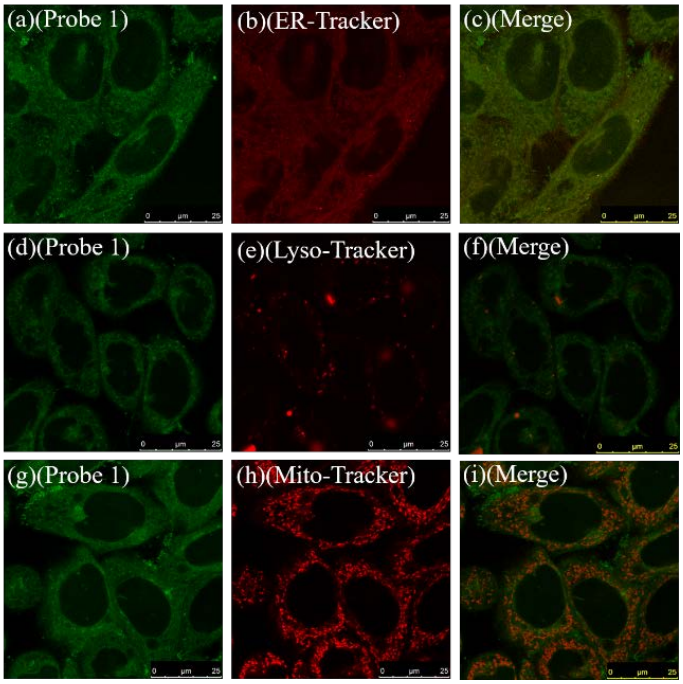


Fig. 4 Fluorescent imaging of probe **1** in HeLa cells; The cells were co-incubating of **1** (1 μM, (a), (d) and (g), Green color, excited at 488 nm, fluorescence collected at 500-550 nm) with ER-, Lyso- and Mito-Trackers [1μM, (b), (e) and (h), Red Color], respectively; merged images [(c), f(f), and (i)] were obtained by the overlapping (a), (d), (g) and (b), (e), (h). For Lyso-tracker, excited at 577 nm, fluorescence collected at 586-659 nm; for ER-tracker, excited at 587 nm, and fluorescence collected at 595-681 nm, and for Mito-Tracker, excited at 581 nm, fluorescence collected at 619-701 nm. For all pictures scale bar = 25 μm.

To observe the interaction between ER and lysosomes during autophagy process; the probe **1** was first incubated with HeLa cells for 1 hour, and then commercial Lyso-tracker was applied to mark the lysosomes inside cells. As demonstrated in Fig. 5, at the beginning, the probe **1** and Lyso-tracker were separately distributed at different organelles (ER and lysosome, respectively). After 1 hour co-incubation of the two probes (Lyso-tracker and probe **1**), some of the probe **1** was found to be located on the same organelles along with Lyso-tracker, implying that ER gradually fused with lysosome upon interaction of **1** with ER. (Fig. 5)

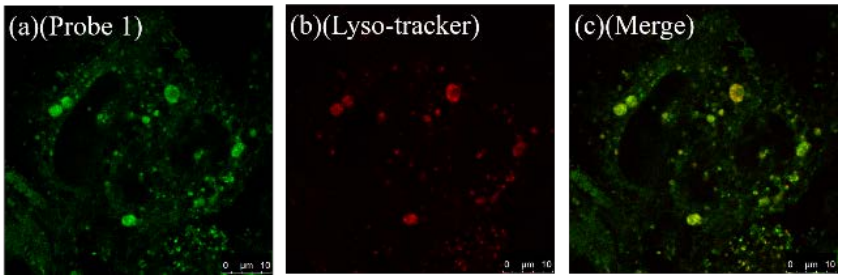


Fig. 5 Colocalization imaging of HeLa cells after 1 hour incubation with probe **1** (1 μM) (a) and Lyso-tracker (b) respectively; for Lyso-tracker, excited at 633 nm, and collected at 645-680 nm.

To further study the microviscosity changes in ER upon incubating **1** with HeLa cells, FLIM imaging microscopy was used to measure the fluorescence lifetime distribution. Fig. 6 clearly indicates that the lifetime was increased from 1.3 to 1.7 ns and the corresponding intracellular viscosity varied from 130 to 200 cP when the viscosity was calculated according to the linear relationship between the lifetime and viscosity as depicted in Fig. 2. It was uncovered that cellular viscosity was markedly increased by the interaction of the probe with live cells. [Fig. 6(a), 6(b)]

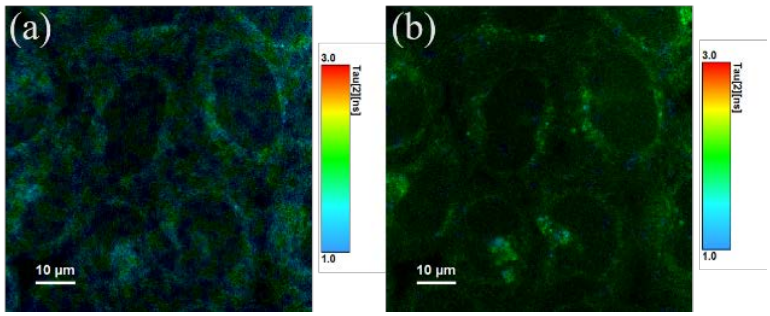


Fig. 6 Fluorescence lifetime imaging (FLIM) of the probe **1** (1μM) in HeLa cells using a time correlated single photon counting system (DCS120). Panel (a) and (b) represents FLIM imaging of **1** in HeLa cells after incubated for 5 min and 1 hour, respectively; excited at 488 nm, and detected at the 520/20 nm.

Furthermore, to prove that the ER autophagy of HeLa cells can be activated by probe **1**, a commercially available chemical of microtubule-associated protein light chain 3B (LC3B) antibody (Abcam, catlog No. ab51520) was employed for co-incubation with **1** in HeLa cells. It

should be mentioned here that LC3B is a well-known mammalian homologue of the autophagy-related Atg8 in yeast, which is often used in autophagy studies.[14-16] After incubated with the probe **1** for 1 hour, as shown in Fig. 7, it was found that LC3B proteins aggregated in the cytoplasm, indicating the occurrence of autophagy [Fig 7(b)]. And it was noticed from the merged figure [Fig.7(c)] that some red autophagosomes of LC3B were overlapped well with the green spots induced by the probe **1**, which suggested that the probe **1** had actually promoted the formation cell autophagy (Fig. 7).

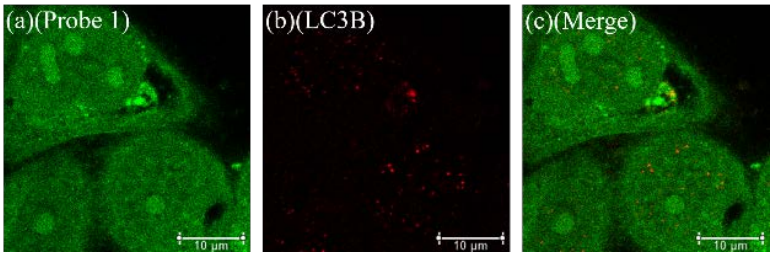


Fig.7 Immunofluorescence imaging of LC3B staining in HeLa cells; Cells were fixed after 1 hour incubation with probe **1**. Panel (a), excited at 488 nm and emission was detected at 500–550 nm by using Leica SP8 confocal microscope. Panel (b), excited at 647 nm and emission was detected at 660–730 nm. Alexa Fluor 647-conjugated Goat to rabbit IgG was used as secondary antibody (Abcam, catalog No. ab150079).

4. Conclusions

In conclusion, a multifunctional fluorescent probe (**1**), capable of biological viscosity quantification and ER autophagy induction was reported to covalently label ER VDPs and to determine local viscosity changes of ER in live cells. Upon incubation of probe with HeLa cells, the binding of complex of **1** with VDPs caused ER stress and subsequently initiated ER autophagy process. In the meanwhile, ER viscosity, which enhanced from ca. 130 to 200 cP, was quantitatively measured using fluorescence lifetime imaging microscopy. This biological process of micro-environmental changes may be referred to many signal pathways. Moreover, this probe

could be used as a promising tool for the diagnosis of ER viscosity, ER stress and autophagy process related diseases.

Disclosures

The authors declare no finance conflicts of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (61525503/21406125/61620106016/81727804); the National Basic Research Program of China (2015CB352005); Guangdong Natural Science Foundation Innovation Team (2014A030312008); Hong Kong, Macao and Taiwan cooperation innovation platform & major projects of international cooperation in Colleges and Universities in Guangdong Province (2015KGJHZ002); and Shenzhen Basic Research Project (JCYJ20150930104948169/JCYJ20160328144746940/GJHZ20160226202139185).

References

[1] Rashid, H. O., Yadav, R. K., Kim, H. R., Chae, H. J., "ER stress: Autophagy induction, inhibition and selection," *Autophagy* **11**(11), 1956-1977 (2015).

[2] Lee, W. S., Yoo, W. H. and Chae, H. J., "ER Stress and Autophagy," *Curr. Mol. Med.* **15**(8), 735-745 (2015).

[3] Molino, D., et al., "ER-driven membrane contact sites: Evolutionary conserved machineries for stress response and autophagy regulation?" *Commun. Integr. Biol*. **10**(5-6): e1401699 (2017).

[4] Kuimova M. K., "Molecular rotors image intracellular viscosity," *Chimia. (Aarau)*. **66**(4), 159-165 (2012).

[5] Lai, C.W., Aronson D. E. and Snapp, E. L., "BiP availability distinguishes states of homeostasis and stress in the endoplasmic reticulum of living cells," *Mol. Biol. Cell.* **21**(12), 1909-1921 (2010).

[6] Yang, Z., He, Y., Lee, J. H. Chae, W.-S., Ren, W. X. Lee, J. H., Kang C. and Kim, J. S., "A Nile Red/BODIPY-based bimodal probe sensitive to changes in the micropolarity and microviscosity of the endoplasmic reticulum," *Chem. Commun.* **50**(79), 11672-11675 (2014).

[7] Yoshida, H., "ER stress and diseases," *FEBS J*. **274**(3), 630-658, (2007).

[8] Huang, H. C., et al., "Endoplasmic reticulum stress as a novel neuronal mediator in Alzheimer's disease," *Neurol. Res.* **37**(4), 366-374 (2015).

[9] Engin, F., et al., "ER stress and development of type 1 diabetes," *J. Investig. Med*. **64**(1), 2-6 (2016).

[10] L. Ozcan and I. Tabas, "Calcium signalling and ER stress in insulin resistance and atherosclerosis," *J. Intern. Med*. **280**(5), 457-464 (2016).

[11] Yang, Z., He, Y., Lee, J.-H., Park, N., Suh, M., Chae, W.-S., Cao, J., Peng, X., Jung, H., Kang, C., Kim, J. S., "A self-calibrating bipartite viscosity sensor for mitochondria," *J. Am. Chem. Soc*. **135**(24), 9181-9185 (2013).

[12] Peng, X., Yang, Z., Wang, J., Fan, J. He, Y., Song, F., Wang, B., Sun, S., Qu, J., Qi, J., and Yan, M., "Fluorescence ratiometry and fluorescence lifetime imaging: using a single molecular sensor for dual mode imaging of cellular viscosity," *Anal. Chem.* **89**(1), 552-555 (2017).

[13]Zhou, K., et al., "Development of a viscosity sensitive fluorescent probe for real-time monitoring of mitochondria viscosity," *New J. Chem*. **41**(20), 11507-11511 (2017).

[14] Wu, J., et al., "Molecular cloning and characterization of rat LC3A and LC3B--two novel markers of autophagosome," *Biochem. Biophys. Res. Commun*. **339**(1), 437-442 (2006).

[15] Mai, S., et al., "Autophagy proteins LC3B, ATG5 and ATG12 participate in quality control after mitochondrial damage and influence lifespan," *Autophagy* **8**(1), 47-62 (2012).

[16] Huang, J., Pan, W., Ou, D., Dai, W., Lin, Y., Chen, Y., and Chen, X., “LC3B, a Protein That Serves as an Autophagic Marker, Modulates Angiotensin II-induced Myocardial Hypertrophy,” *J. Cardiovasc. Pharmacol*. **66**(6), 576-583 (2015).