Fluorescence life-time imaging microscopy (FLIM) monitors tumor cell death triggered by photothermal therapy with MoS₂ nanosheets

Hongda Liang†, Zheng Peng†, Xiao Peng*, Yufeng Yuan, Teng Ma, Yiwan Song, Jun Song and Junle Qu

Key Laboratory of Optoelectronic Devices and Systems of Ministry of Education and Guangdong Province
College of Physics and Optoelectronic Engineering
Shenzhen University, Shenzhen 518060, P. R. China
*pengxiao_px@szu.edu.cn

Received 14 March 2019
Accepted 6 May 2019
Published 26 June 2019

Recently, photothermal therapy (PTT) has been proved to have great potential in tumor therapy. In the last several years, MoS₂, as one novel member of nanomaterials, has been applied into PTT due to its excellent photothermal conversion efficacy. In this work, we applied fluorescence lifetime imaging microscopy (FLIM) techniques into monitoring the PPT-triggered cell death under MoS₂ nanosheet treatment. Two types of MoS₂ nanosheets (single layer nanosheets and few layer nanosheets) were obtained, both of which exhibited presentable photothermal conversion efficacy, leading to high cell death rates of 4T1 cells (mouse breast cancer cells) under PTT. Next, live cell images of 4T1 cells were obtained via directly labeling the mitochondria with Rodamine123, which were then continuously observed with FLIM technique. FLIM data showed that the fluorescence lifetimes of mitochondria targeting dye in cells treated with each type of MoS₂ nanosheets significantly increased during PTT treatment. By contrast, the fluorescence lifetime of the same dye in control cells (without nanomaterials) remained constant after laser irradiation. These findings suggest that FLIM can be of great value in monitoring cell death process during PTT of cancer cells, which could provide dynamic data of the cellular microenvironment at single cell level in multiple biomedical applications.

Keywords: Fluorescence lifetime imaging microscopy (FLIM); MoS₂ nanosheets; photothermal therapy (PTT); 4T1 cells.

*Corresponding author.
†Hongda Liang and Zheng Peng contributed equally to this work and are considered as co-first authors.
1. Introduction

At present, cancer is one of most lethal diseases in the world. There are still great limitations of traditional treatment strategies including surgery, chemotherapy, and radiotherapy, leading to development of a lot of alternative novel therapeutic approaches. Among these various treatment approaches, photothermal therapy (PTT), which employs heat generated from visible or near infrared (NIR) absorbed materials to ablate tumors, has attracted more and more attention due to its advantages including minimal invasiveness and high efficiency against drug-resistant tumors. In the last several years, two-dimensional (2D) molybdenum disulfide (MoS2) nanomaterials have shown high light-absorption ability and satisfactory biocompatibility, making them potential powerful PTT agents for tumor treatment via their NIR absorption properties.

At present, the standard methods for measuring the efficiency of the PTT agents against tumor cells includes biochemical methods such as cytotoxicity assays and microscopy methods such as live and dead cell imaging assays. However, these methods could not provide dynamic information of cell death at single cell level or even at subcellular level. Therefore, the subcellular processes of PTT triggered cell death still needs further investigation. Compared with the above-mentioned methods, fluorescence lifetime imaging microscopy (FLIM) technique has great advantages in monitoring multiple cell physiological processes, including cell growth and cell death, since FLIM can provide real-time images and quantitative subcellular information.

In this study, we applied FLIM method to observe MoS2 nanosheet treated 4T1 cells in order to study the mitochondria damage during cell death. We used two types of MoS2 nanosheets, which are single layer nanosheets and few layer nanosheets (abbreviated as SL and FL, respectively), and obtained their photothermal conversion efficacy. Then, we confirmed their PTT efficacy on 4T1 cells under 808 nm laser irradiation. After the cell mitochondria were labeled with Rodamine123 (abbreviated as R123) dye, cells incubated with MoS2 nanosheets or control cells under 808 nm laser irradiation were respectively monitored by using FLIM technique. Finally, FLIM data were analyzed to evaluate the antitumor efficacy at single cell level.

2. Methods

2.1. Chemicals

Both of the MoS2 single layer nanosheets and few layer nanosheets were purchased from Nanjing XFNANO Materials Tech. Rodamine123 were purchased from Beyotime Biotechnology. Cell cytotoxicity kits (CCK-8) were obtained from Nanjing KeyGen Biotech. All the cell culture reagents (medium, antibiotics and fetal bovine serum) were obtained from Invitrogen.

2.2. Material characterizations

The morphology of the MoS2 nanosheets was obtained by a transmission electron microscopy (TEM) instrument (JEM-1230 CX, Jeol Ltd, Japan). The UV-Vis-NIR spectra of these nanosheets were recorded in a 10 mm path length cuvette on a spectrophotometer (UV1780, Shimadzu, Japan). The OD values in CCK-8 assays were determined by a microplate reader (Infinite M200, Tecan, Switzerland).

2.3. Photothermal properties of the MoS2 nanosheets

To evaluate the photothermal properties of the MoS2 nanosheets, a series of MoS2 aqueous solutions at different concentrations (0, 25, 50, 100, 200 or 400 µg/mL) were prepared and deposited into cuvettes. Then the cuvettes were illuminated by a continuous-wave laser (808 nm) at a power density of 1 W/cm². The temperatures were continuously recorded by using a probe thermometer at every 30 s (for heating period) or every 1 min (for cooling period).

The photothermal conversion efficiency PTCE (η) was calculated as mentioned before as follows:

$$\eta = \frac{h A \Delta T_{\text{max}} - Q_s}{I(1 - 10^{-A_{808}})}.$$  \hspace{1cm} (1)

In Eq. (1), \(h\) represents the heat transfer coefficient. \(A\) is the container surface area (1 cm²). \(\Delta T_{\text{max}}\) is the maximum temperature change of the nanosheet solution at the corresponding time point. \(Q_s\) is defined as the heat loss due to the light absorbed by the container. \(I\) refers to the incident power density of the 808 nm laser (1 W/cm²) and A 808 is the absorbance value of the solution at 808 nm.
In order to get the $hA$ in Eq. (1), a parameter $\theta$ is introduced as shown in Eq. (2), which is defined as the ratio of $\Delta T$ to $\Delta T_{\text{max}}$.

$$\theta = \frac{\Delta T}{\Delta T_{\text{max}}}.$$  \hspace{1cm} (2)

$\Delta T$ stands for the temperature change during a cooling period. In addition, $hA$ is calculated by applying the linear time data from the cooling period versus $\ln \theta$.

### 2.4. Cell culture and cytotoxicity assay

4T1 cells (mouse breast cancer cells) were purchased from American Type Culture Collection (ATCC, USA). Cells were cultured in DMEM culture medium with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37°C under a 5% CO$_2$ and humidity condition.

In order to examine the biocompatibility of the MoS$_2$ nanosheets, a standard CCK-8 cell assay was carried on 4T1 cells. Around 10,000 of cells were seeded in each well of 96 well plates and grown overnight. The next day, medium with different concentrations of the MoS$_2$ nanosheets (50, 100 or 200 $\mu$g/mL) were added to the cells. Here, six replicates were applied for each condition. After 24 h incubation, the solutions were moved from cells and a mixture of 10 $\mu$L of CCK-8 solution plus 100 $\mu$L of fresh culture medium was added to each well, followed by another incubation for around 1–2 h. At the end of the chromogenic reaction, the OD value at 450 nm wavelength of each well of the plates was recorded by using the microplate reader.

Next, the PTT efficiency of the MoS$_2$ nanosheets was measured by the CCK-8 assay in a similar way. Briefly, 4T1 cells were seeded in 96-well plates and cultured overnight. Then, PBS or MoS$_2$ nanosheets (0, 100 or 200 $\mu$g/mL) were added to the medium, and the cells were incubated in 5% CO$_2$ at 37°C. After incubation, the PBS or MoS$_2$ nanosheet treated samples (PTT groups) were illuminated under 808 nm laser irradiation (1 W/cm$^2$) for 5 mins, followed by the CCK-8 assay at the same procedure mentioned above.

### 2.5. FLIM experiments

In order to obtain FLIM images, around 100,000 of cells were seeded in each 35 mm confocal dish. After an overnight incubation, the cells were labeled with R123 and then incubated in fresh medium with either PBS or MoS$_2$ nanosheets (200 $\mu$g/mL). These cell dishes were then moved onto the microscope stage and observed for 18 mins. The images were obtained at every 3 mins during this period. Next, the image acquisition was continuously performed at the same frequency while introducing the 808 nm laser irradiation (1 W/cm$^2$) onto the cells for another 15 mins.

All microscopic imaging and FLIM analyses were performed with a Nikon Eclipse confocal system mounted on an inverted microscope. This microscope was equipped with a Fianium whitelase Supercontinuum laser (output laser from 470 to 670 nm, 6 ps pulse width, 80 MHz repetition rate) for single photon excitation. A 63 x oil immersion objective lens (NA 1.40) was used for all images. To obtain FLIM data, the time-correlated single-photon counting (TCSPC) technique was carried out with a Becker & Hickl SPC120 unit. In this study, the 514 nm excitation wavelength was selected to generate the fluorescence images of cells labeled with R123. A band-pass filter BP 535/30 was put in front of the PMT detector to acquire the specific signals. All FLIM images were acquired at 256 x 256 pixels and the collection time for one image was around 30 s. After image collection, the fluorescence lifetime image data was analyzed by using SPICEImage (Becker & Hickl) software as described in our former studies, providing the fluorescence decay signal of each image and lifetimes of the selected regions.

Next, to evaluate the photostability of R123 dye under NIR light irradiation, we carried out the following experiments. Briefly, the R123 solution (50 $\mu$M) or the cells labeled with R123 were prepared in confocal dishes. Then the dishes were moved onto the microscope stage and observed for 18 mins. The images were obtained at every 3 mins during this period and the photon numbers collected in 1 min were counted at each timepoint. After the 808 nm laser irradiation (1 W/cm$^2$) was turned on, the photon-counting image acquisition was continuously carried out for extra 15 mins.

In addition, we used FLIM technique to investigate the parameters that might influence the fluorescence lifetime of R123 dye. The R123 dye samples were prepared in a series of solutions (100 $\mu$M) with different pH values or with different viscosities. Then these two series of R123 dye solutions were measured with FLIM system, providing images for data analysis.
3. Results and Discussion

3.1. Characterization of MoS$_2$ nanosheets

In this study, both MoS$_2$ nanosheets are stored in aqueous solutions. As shown in the TEM image (Figs. 1(a) and 1(b)), a general view of the MoS$_2$ nanosheets showed that the lateral sizes of MoS$_2$ SL nanosheets and FL nanosheets are both in the range from around 50 to 200 nm, which is a suitable size range for efficient endocytosis in the applications of nanoparticles into biological fields.$^{21,22}$

Moreover, the absorption range of both of the two types of MoS$_2$ nanosheets with the gradient concentrations (25, 50, 100, 200 or 400 $\mu$g/mL) expand to the NIR region from 700 to 900 nm (Figs. 1(c) and 1(d)), which is very similar to the results reported before.$^{9-13}$ This range is compliant with an 808 nm laser in the further investigation on their photothermal properties.

3.2. Photothermal properties of MoS$_2$ nanosheets

In order to examine the photothermal properties of MoS$_2$ SL nanosheets and FL nanosheets, we recorded the temperature trends of the aqueous solutions with a series of concentrations (0, 50, 100, and 200 $\mu$g/mL) that were exposed to an 808-nm laser irradiation (1 W/cm$^2$) for 5 mins.

As shown in Figs. 2(a) and 2(b), the solution temperatures rapidly increase in 5 mins, and the

Fig. 1. (a)–(b) TEM images of MoS$_2$ single-layer nanosheets (a) and few-layer nanosheets (b), respectively. Scalebar: 100 nm. (c) and (d) Absorbance spectra of MoS$_2$ SL (c) and FL (d) nanosheets with the gradient concentrations, respectively.
slopes are positively associated with the increased concentrations of nanomaterials. For example, the temperature of the MoS$_2$ SL nanosheet solution (200 $\mu$g/mL) dramatically increased from around 23.0°C to 61.8°C in 5 mins, achieving an excellent temperature high enough to an irreversible ablation to the tumor cells. Under the same condition, the temperature change of the MoS$_2$ FL nanosheet solution (200 $\mu$g/mL) exhibited even a greater increase to around 80°C after laser irradiation for 5 mins, which indicates the higher photothermal transduction efficacy of MoS$_2$ FL than that of MoS$_2$ SL nanosheets.

To further investigate the photothermal transduction ability of these two types of nanosheet aqueous solution, we measured the temperature profiles of each solution with a concentration of 200 $\mu$g/mL under a continuous laser irradiation (1 W/cm$^2$) for 5 mins, consequently followed by a natural cooling period till room temperature. The temperature profile results were shown as in Figs. 2(c) and 2(d). These data were analyzed according to the reported method, resulting in the photothermal conversion efficiency ($\eta$) of the MoS$_2$ SL and the MoS$_2$ FL nanosheets, which could achieve 17.4% and 24.8%, respectively, similar to that of previously reported MoS$_2$ nanosheets (24.37% or 26.7%). Such suitable photothermal conversion capabilities indicate that MoS$_2$ nanosheets could offer great advantages for PTT.

Fig. 2. (a)–(b) Heating curves of MoS$_2$ SL nanosheets (a) or MoS$_2$ FL nanosheets (b) aqueous solution with a series of concentrations during irradiation (808 nm, 1 W/cm$^2$). (c)–(d) The temperature profile of the MoS$_2$ SL nanosheets (c) or MoS$_2$ FL nanosheets (d) under irradiation for 5 mins (808 nm, 1 W/cm$^2$) and the following cooling period (turning off the laser). The insets are the relationship between $-\ln(\theta)$ and time of MoS$_2$ SL nanosheets (c) or MoS$_2$ FL nanosheets (d), which were calculated from the cooling curves, respectively.
3.3. **Photothermal therapy in vitro**

In order to evaluate the potential PTT application abilities of MoS2 SL or FL nanosheets, we carried out cell cytotoxicity assay as well as PTT effect assay on 4T1 cells. Firstly, the potential toxicity of MoS2 SL or MoS2 FL was measured with a CCK-8 assay as the manufacture’s instructions. Experimental data showed that there was little cytotoxicity for either MoS2 SL or FL nanosheets after 24 h incubation, even at the highest concentration 200 µg/mL (Fig. 3(a)), indicating their good biological compatibility.

In addition, we examined the effect of the PTT of MoS2 SL or FL nanosheets in 4T1 cells by an exposure to the 808 nm laser irradiation. As presented in Figs. 3(b) and 3(c), in the PBS, only nanosheets, or only NIR (808 nm, 1 W/cm², 5 mins) treated group, all the cell viabilities reached more than 90%, suggesting negligible damage effects. By contrast, in the MoS2 SL or FL nanosheets plus NIR irradiation group, more than 80% of the cells were killed due to the nanosheet induced PTT effects, which were consistent with former studies.9,17

3.4. **FLIM data acquisition and analysis**

Finally, we performed FLIM experiments for measuring the PTT effects of MoS2 SL or FL nanosheets on 4T1 cells to acquire dynamic subcellular information. In order to monitor the local molecular environment changes in live 4T1 cells, the fluorescent dye R123 was used as a mitochondria localization marker in this process.24,25 During each experiment, around 10–20 of live 4T1 cells were selected for continuously monitoring the fluorescence lifetime of R123 in cell mitochondria. The fluorescence lifetime image of these cells was obtained for every 3 mins in the first period (no irradiation). Then an 808 nm laser was turned on to

![Graphs](image)

Fig. 3. (a) Cell viability of 4T1 cells after incubation with different concentrations of MoS2 SL or MoS2 FL nanosheets after 24 h incubation. (b)–(c) Cell viability of 4T1 cells treated with MoS2 SL (b) or MoS2 FL (c) nanosheets with or without laser irradiation (808 nm, 1 W/cm², 5 mins). Data are shown as mean ± SEM.
irritate the cells at 1 W/cm². We recorded the fluorescence lifetime of R123 for 18 mins and then kept recording for another 15 mins after laser on.

Figure 4(a) were a series of fluorescence lifetime images of typical 4T1 cells during laser irradiation, representing the R123 fluorescence lifetime distributions of the dye in the cell mitochondria. Figure 4(b) was one representative raw result that collected the cells treated with MoS₂ FL nanosheets under NIR laser irradiation at 15 mins. This figure includes the fluorescence intensity image which indicates suitable signal intensity, the fluorescence lifetime image and the histogram of the lifetime distribution of the fluorescent R123 dye in the whole image. The inset in the lower part provides a decay curve for calculating the fluorescence lifetime. As shown in Fig. 4(c), the R123 fluorescence lifetime from cells treated with both types of MoS₂ nanosheets showed slight fluctuations during the first 18 mins before laser on, indicating the nanosheet incubation had no effect on the fluorescence lifetime values. From the initial of laser irradiation, the fluorescence lifetimes of R123 in cells treated with both MoS₂ nanosheets significantly increased in an irreversible way. By contrast, the fluorescence lifetime of the same dye in the control cells remained constant. These above data showed that under physiological conditions, the fluorescence lifetimes of R123 in healthy 4T1 cells could keep constant with random fluctuation. The values of such fluctuation are usually in the range of 50–200 ps. The induction of NIR laser exposure onto nanosheet treated 4T1 cells lead to around 500 ps increases the obtained fluorescence lifetimes suggesting the significant correlation between the cell death and the PTT effects.

Moreover, the photostability of R123 were tested by exposing the R123 solution or the cells labeled with R123 under NIR laser irradiation. Both in vitro and in vivo results are shown in Figs. 5(a) and 5(b), respectively. The collected photon number of the in vitro sample keeps almost constant while the photon number from in vivo sample maintains around 90% after laser explosion, suggesting the satisfactory photostability of R123 through the whole experiment processes.

To further understand the potential microenvironment parameters that might influence the fluorescence lifetimes of R123, the R123 dye samples in different solutions were then measured with FLIM.
system. The images were then obtained and analyzed, providing the data in Figs. 5(c) and 5(d). The fluorescence lifetimes of R123 fluctuated slightly at different pH conditions (Fig. 5(c)). By contrast, as shown in the Fig. 5(d), the R123 decreased as the percentage of glycerol in glycerol-water solutions increased from 0% to 80%, indicating that the fluorescence lifetime of R123 might be sensitive to the change of viscosity.

R123 has been considered as a satisfactory live-cell probe for decades, which could target mitochondria due to its property of probing transmembrane potential.\(^{24,25}\) In this work, when the 4T1 cells were killed during PTT procedures, the transmembrane potential of mitochondria might decline upon cells death, leading to the release of R123 dye from mitochondria into cytoplasm. As a result, the altered microenvironment might cause the changes of the R123 fluorescence lifetime. Therefore, these results suggest that FLIM could provide real-time quantitative data in monitoring PTT triggered cancer cell death.

4. Summary

In this study, MoS\(_2\) SL and FL nanosheets have been used for PTT in 4T1 cells. The NIR photothermal conversion efficiency of MoS\(_2\) SL and FL nanosheets reach the satisfactory values, ensuring them strong capabilities as powerful PTT agents. The CCK-8 data show that both the nanosheets have little toxicity and great cell killing efficacy to 4T1 cells. In addition, FLIM techniques are used to monitor the local environment changes of the mitochondria compartments in live 4T1 cells. The significant fluorescence lifetime changes in mitochondria of the nanosheet treated cancer cells have been proved to be quantitative measurement.
methods for representing PTT induced cell death in a non invasive way. Currently, the organelle-targeting and image-guided therapy has attracted more and more research interests. Therefore, this work is expected to expand the utility of FLIM for specific subcellular microenvironments into evaluating and examining the development of novel tumor thermo-chemo therapies.

Conflict of Interest
The authors have declared that no competing interest exists.

Acknowledgments
This work was supported by the National Key R&D Program of China (2018YFC0910602), the National Natural Science Foundation of China (Grant Nos. 61775145/61835009/81727804), the National Natural Science Foundation of Guangdong Province (Grant Nos. 31771584/61775145/61605121, 61620106016/61525503/61835009/81727804), Guangdong Natural Science Foundation Innovation Team (2014A030312008), and Shenzhen Basic Research Project (JCYJ2017010180153423/JCYJ2017041210212234/JCYJ2016032814474940/JCYJ20170412105005320/JCYJ2017030214290581), Science Foundation of SZU (Grant No. 000193).

References
1. C. J. Lord, A. Ashworth, “The DNA damage response and cancer therapy,” Nature 481, 287–294 (2012).
2. W. Landier, “Ototoxicity and cancer therapy,” Cancer 122, 1647–1658 (2016).
3. S. Vijayakumar, S. Selvakumar, Y. Chen-Sheng, “Near-infrared light-responsive nanomaterials in cancer therapeutics,” Chem. Soc. Rev. 43, 6254–6267 (2014).
4. H. Li, P. Wang, Y. Deng, M. Zeng, Y. Tang, W. H. Zhu, Y. Cheng, “Combination of active targeting, enzyme-triggered release and fluorescent dye into gold nanoclusters for endomicroscopy-guided photothermal/photoradiation therapy to pancreatic ductal adenocarcinoma,” Biomaterials 139, 30–38 (2017).
5. J. J. Hu, Y. J. Cheng, X. Z. Zhang, “Recent advances in nanomaterials for enhanced photothermal therapy of tumors,” Nanoscale 10, 22657–22672 (2018).
6. Y. Chen, Z. Fan, Z. Zhang, W. Niu, C. Li, N. Yang, B. Chen, H. Zhang, “Two-dimensional metal nanomaterials: FLIM monitors PTT triggered cell death and synergistic chemo-photothermal therapy by monodispersed-MoS2-nanosheets wrapped periodic mesoporous organosilicas,” J. Mater. Chem. B 4, 7708–7771 (2016).
7. T. Fan, Y. Zhou, M. Qiu, H. Zhang, “Black phosphorus: A novel nanoplateform with potential in the field of bio-photonic nanomedicine,” JIOHS 11, 1830009 (2018).
8. C. Murugan, V. Sharma, R. K. Murugan, G. Malaimegu, A. Sundaramurthy, “Two-dimensional cancer theranostic nanomaterials: Synthesis, surface functionalization and applications in photothermal therapy,” J. Control. Release 299, 1–20 (2019).
9. W. Yin, L. Yan, J. Yu, G. Tian, L. Zhou, X. Zheng, X. Zhang, Y. Yong, J. Li, Z. Gu, “High-throughput synthesis of single-layer MoS2 nanosheets as a near-infrared photothermal-triggered drug delivery for effective cancer therapy,” ACS Nano 8, 6922–6933 (2014).
10. T. Liu, C. Wang, X. Gu, H. Gong, L. Cheng, X. Shi, L. Feng, B. Sun, Z. Liu, “Drug delivery with PEGylated MoS2 nano-sheets for combined photothermal and chemotherapy of cancer,” Adv. Mater. 26, 3433–3440 (2014).
11. T. Shao, J. Wen, Q. Zhang, Y. Zhou, L. Liu, L. Yuwen, Y. Tian, Y. Zhang, W. Tian, Y. Su, Z. Teng, G. Lu, J. Xu, “NIR photoresponsive drug delivery and synergistic chemo-photothermal therapy by monodispersed-MoS2-nanosheets wrapped periodic mesoporous organosilicas,” J. Mater. Chem. B 4, 7708–7771 (2016).
12. L. Kong, L. Xing, B. Zhou, L. Du, X. Shi, “Dendrimer-modified MoS2 nanoflakes as a platform for combinational gene silencing and photothermal therapy of tumors,” ACS Appl. Mater. Interf. 9, 15995–16005 (2017).
13. S. Gao, H. Zhou, S. Cui, H. Shen, “Bottom-up synthesis of MoS2 nanospheres for photothermal treatment of tumors,” Photochem. Photobiol. Sci. 17, 1337–1345 (2018).
14. S. M. Levchenko, A. Pliss, J. Qi, “Fluorescence lifetime imaging of fluorescent proteins as an effective quantitative tool for noninvasive study of intracellular processes,” JIOHS 11, 1730009 (2018).
15. S. Suarasan, A. M. Craciun, E. Licarete, M. Focsan, K. Magyari, S. Astilean, “Intracellular dynamic disentangling of doxorubicin release from luminescent cyclodextrin gold carriers by fluorescence lifetime imaging microscopy (FLIM) under two-photon excitation,” ACS Appl. Mater. Interf. 11, 7812–7822 (2019).
16. D. K. Roper, W. Ahn, M. Hoepfner, “Microscale heat transfer transduced by surface Plasmon resonant gold nanoparticles,” J. Phys. Chem. C Nanomater Interf. 111, 3636–3641 (2007).
17. Q. Wu, Y. Lin, F. Wo, Y. Yuan, Q. Ouyang, J. Song, J. Qu, K. T. Yong, “Novel magnetic-luminescent janus nanoparticles for cell labeling and tumor photothermal therapy,” *Small* **13**, 1701129 (2017).

18. A. Pliss, X. Peng, L. Liu, A. Kuzmin, Y. Wang, J. Qu, Y. Li, P. N. Prasad, “Single cell assay for molecular diagnostics and medicine: Monitoring intracellular concentrations of macromolecules by two-photon fluorescence lifetime imaging,” *Theranostics* **5**, 919–930 (2015).

19. S. Zhou, X. Peng, H. Xu, Y. Qin, D. Jiang, J. Qu, H. Y. Chen, “Fluorescence lifetime-resolved ion-selective nanospheres for simultaneous imaging of calcium ion in mitochondria and lysosomes,” *Anal. Chem.* **90**, 7982–7988 (2018).

20. A. Pliss, S. M. Levchenko, L. Liu, X. Peng, T. Y. Ohulchanskyy, I. Roy, A. N. Kuzmin, J. Qu, P. N. Prasad, “Cycles of protein condensation and discharge in nuclear organelles studied by fluorescence lifetime imaging,” *Nat. Commun.* **10**, 455 (2019).

21. W. Jiang, B. Y. Kim, J. T. Rutka, W. C. Chan, “Nanoparticle-mediated cellular response is size-dependent,” *Nat. Nanotechnol.* **3**, 145–150 (2008).

22. H. Cabral, Y. Matsumoto, K. Mizuno, Q. Chen, M. Murakami, M. Kimura, Y. Terada, M. R. Kano, K. Miyazono, M. Uesaka, N. Nishiyama, K. Kataoka, “Accumulation of sub-100 nm polymeric micelles in poorly permeable tumours depends on size,” *Nat. Nanotechnol.* **6**, 815–823 (2011).

23. S. N. Goldberg, G. S. Gazelle, P. R. Mueller, “Thermal ablation therapy for focal malignancy: A unified approach to underlying principles, techniques, and diagnostic imaging guidance,” *Am. J. Roentgenol.* **174**, 323–331 (2000).

24. R. K. Emaus, R. Grunwald, J. J. Lemasters, “Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: Spectral and metabolic properties,” *Biochim. Biophys. Acta.* **850**, 436–448 (1986).

25. H. Schneckenburger, K. Stock, M. Lyttek, W. S. Strauss, R. Sailer, “Fluorescence lifetime imaging (FLIM) of rhodamine 123 in living cells,” *Photochem. Photobiol. Sci.* **3**, 127–131 (2004).

26. J. Zhang, Q. Wang, Z. Guo, S. Zhang, C. Yan, H. Tian, W. H. Zhu, “High-fidelity trapping of spatial-temporal mitochondria with rational design of aggregation-induced emission probes,” *Adv. Funct. Mater.* **29**, 1808153 (2019).