

# Synthesis and characterization of a novel near-infrared fluorescent probe for applications in imaging A549 cells

Jing Xing · Gaoxin Zhou · Chunlong Sun · Huanqing Zhang ·  
Bo Chen · Xi Zong · Jin Cai · Min Ji

Received: 9 January 2016 / Accepted: 19 July 2016 / Published online: 2 August 2016  
© Springer Science+Business Media Dordrecht 2016

## Abstract

**Objective** To design and synthesize a novel near-infrared (NIR) fluorescent probe based on indocyanine Green (ICG), that can be applied in imaging living cells.

**Results** A highly fluorescent novel NIR fluorescent probe (IR-793) was synthesized in two steps. IR-793 had better fluorescence and optical stability than ICG. In addition, no obvious cytotoxicity effect of IR-793 was observed and cell viability was above 75% at the maximum concentration (120 nM). IR-793 also exhibited good performance in imaging living A549 cells.

**Conclusion** IR-793, a novel NIR fluorescent probe that is stable, low-cost, highly fluorescent and low cytotoxicity, has been designed and synthesized for imaging living cells.

**Keywords** Fluorescent dye · Fluorescence imaging · IR-793 · Imaging of living cells · Indocyanine Green · Living cells · A549 cells

## Introduction

Near-infrared (NIR) fluorescence imaging is a powerful tool in cell biology, pharmacology, and disease diagnosis (Mather 2009). NIR probes fluoresce from 600 to 900 nm, which can decrease background fluorescence and allow deep tissue penetration (Zhang et al. 2010). Therefore, NIR fluorescent probes are applied in disease diagnosis and biomarker development (Rasmussen et al. 2009).

At present, only a few NIR imaging probes have received approval for clinical use from FDA (Luo

**Electronic supplementary material** The online version of this article (doi:10.1007/s10529-016-2179-z) contains supplementary material, which is available to authorized users.

J. Xing · G. Zhou · B. Chen · M. Ji (✉)  
School of Biological Science & Medical Engineering,  
Southeast University, Nanjing 210096, China  
e-mail: seujimin@126.com

J. Xing · G. Zhou · B. Chen · M. Ji  
School of Biological Science and Medical Engineering &  
Collaborative Innovation Center of Suzhou Nano Science  
and Technology, Southeast University, Suzhou 215123,  
China

C. Sun  
College of Life Sciences, Binzhou University,  
Binzhou 256600, China

H. Zhang  
Chia-tai Tianqing Pharmaceutical Group Co. Ltd.,  
No.699-8 Xuanwu Blvd., Nanjing 210042, China

X. Zong · J. Cai  
School of Chemistry & Chemical Engineering, Southeast  
University, Nanjing 210096, China

et al. 2011). Indocyanine Green (ICG), which is one of the NIR fluorescent probes approved by FDA, has low toxicity, negligible side effects, and is widely used for monitoring cardiac function, hepatic output, and retinal angiography. In addition, ICG has been recently used as a potential photosensitizer (Mamoon et al. 2009; Funayama et al. 2012; Sevic-Muraca 2012). However, the applications of ICG have several limitations, including low quantum yield, optical instability in the body, and unrestrained leakage in blood vessels. Thus, many research groups have focused on the synthesis of ICG derivatives to improve the brightness, solubility, photostability, and optical stability for both in vitro and in vivo imaging (Xu et al. 2007; Chang et al. 2008).

We have designed and synthesized various ICG derivatives as NIR imaging probes for non-invasive in vivo imaging research. A series of NIR fluorescence probes, namely, IR-780, IR-789, and MP-Cy, which exhibit high sensitivity, good photostability, and excellent cell membrane permeability, have been identified by our laboratory (Sun et al. 2013, 2014, 2015). In the current work, considering the aforementioned limitations of ICG, we have designed and synthesized a novel NIR fluorescent probe IR-793 which has better water solubility and optical stability in serum when applied in cellular analysis in an aqueous environment. Herein, we describe IR-793, a NIR fluorescent dye with high extinction coefficients, can provide good photostability with high extinction coefficients and show promising application in biomedical diagnosis.

## Materials and methods

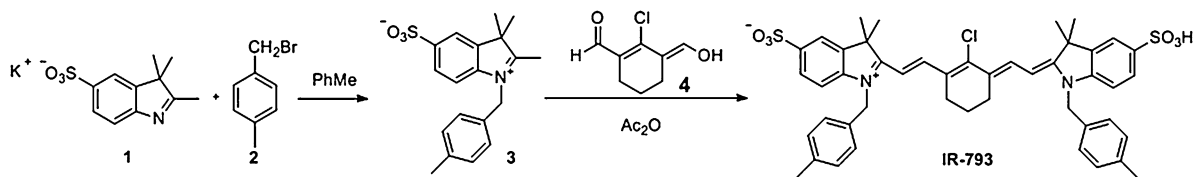
### Materials and instruments

<sup>1</sup>H-NMR data were recorded using an ACF-300/500 MHz NMR spectrometer (Bruker) under ambient temperature in DMSO-d<sub>6</sub>, and tetramethylsilane was

used as internal standard reference. Molecular weight was determined using a LC/MS system (Agilent). 2,3,3-Trimethylindolenine-5-sulfonic acid, *p*-methylbenzyl bromide, 2-chloro-1-formyl-3-hydroxymethylcyclohexene, and ICG were purchased from Sigma-Aldrich (Shanghai). The A549 cell line (human lung adenocarcinoma epithelial cell line) was obtained from ATCC. All reagents and solvents were commercially available and used without further purification, unless otherwise indicated.

### Synthesis and characterization of IR-793

Scheme 1 shows the synthetic route of the designed heptamethine cyanine dye IR-793. Compound 1, 2,3,3-trimethylindolenine-5-sulfonic acid (0.68 g, 2.45 mmol), and compound 2, *p*-methylbenzyl bromide (0.54 g, 2.96 mmol), were dissolved in 10 ml toluene. The solution was stirred under argon for 12 h at 85 °C. Vacuum filtration was performed to isolate the resulting precipitate and obtain a crude solid that was recrystallized to yield compound 3, which is a pink solid (0.70 g, yield 83.1 %). Compound 3, 1-*p*-methylbenzyl bromide-2,3,3-trimethylindolenine-5-sulfonic acid (0.65 g, 1.90 mmol), compound 4, 2-chloro-1-formyl-3-hydroxymethylcyclohexene (0.16 g, 0.95 mmol), and anhydrous sodium acetate (0.90 mmol, 0.16 g) were dissolved in 16 ml acetic anhydride. The solution was stirred under argon for 1 h at 75 °C. The resulting solution was added into the methyl tert-butyl ether to yield a crude solid. The crude residue was purified via column chromatography on silica gel using dichloromethane/methanol (10:1, R<sub>f</sub> = 0.40), which yielded IR-793 (240 mg, 30.8 %) as a dark green solid. The IR-793 fractions of high chemical purity were collected and tested through <sup>1</sup>H-NMR spectroscopy using ACF-300/500 MHz NMR spectrometers. Then, the compound of IR-793 was further evaluated with a quadrupole time-of-flight LC/MS system.



**Scheme 1** Synthetic route of heptamethine cyanine dye IR-793

### Absorption spectra

Absorption spectra were obtained using a UV–Vis spectrophotometer at room temperature and within the spectral range of 600–900 nm. Probe IR-793 (1 ml, 0.01 mM) and ICG (1 ml, 0.01 mM) were studied via absorption spectroscopy in PBS, methanol solutions, or serum.

### Fluorescence spectra

Fluorescence spectra were determined using a spectrofluorimeter. Probe IR-793 (0.1 ml, 0.01 mM) and ICG (0.1 ml, 0.01 mM) were separately added into the 1 ml color comparison tubes and studied through fluorescence spectroscopy in PBS, methanol solutions, or serum.

### Optical stability

The optical stability of IR-793 in serum was determined through the previously reported method that used fluorescence spectroscopy (799 nm excitation, continuous wavelength of 600–900 nm emission) (Nakayama et al. 2003; Zhang et al. 2010). A 10  $\mu\text{M}$  IR-793 was incubated in fetal bovine serum at 37 °C for 2 h to evaluate the stability in serum. Fluorescence intensities were detected in 10 ml color comparison tubes using a Cary Eclipse spectrofluorometer (740 nm excitation, 825 nm emission) every 10 min for 1 h, then at 2 h. All the experiments were performed in triplicates, and data were plotted as mean values  $\pm$  SD.

### Cell culture

Human lung cancer cells (A549) were cultured in RPMI-1640 supplemented with 10 % (v/v) fetal calf serum, 1 % penicillin, and 1 % streptomycin. Cells were grown routinely in a monolayer culture at 37 °C under 5 % CO<sub>2</sub> humidified atmosphere in T25 cell culture flasks. Cells were split in 1:3 ratios at 80 % confluence.

### Cytotoxicity assay

MTT assay was conducted to assess the cytotoxicity of IR-793. The A549 cells (5000 cells per well) were seeded in 96-well plates for 24 h before

treatment. The culture medium in each well was replaced with 200  $\mu\text{l}$  IR-793-supplemented treatment medium. Various concentrations of 10, 20, 40, 60, 80, and 120 nm IR-793 were tested. A medium without IR-793 was used as the control. Cells were further incubated for 24 and 48 h. After the incubation period, the cells were washed thrice with PBS before adding 100  $\mu\text{l}$  MTT working solution at 0.5 mg ml<sup>-1</sup>, followed by incubation for 4 h at 37 °C with 5 % (v/v) CO<sub>2</sub>. The remaining MTT solution was removed after 4 h, and 100  $\mu\text{l}$  DMSO was added into each well to dissolve the formazan crystals, followed by incubation at 37 °C with 5 % CO<sub>2</sub> for 15 min. The absorbance of each well at 490 nm was recorded using a plate reader. Cell viability was determined by obtaining the signal from the treated groups over the control groups without irradiation. All experiments were performed in triplicates, and data were plotted as mean values  $\pm$  SD.

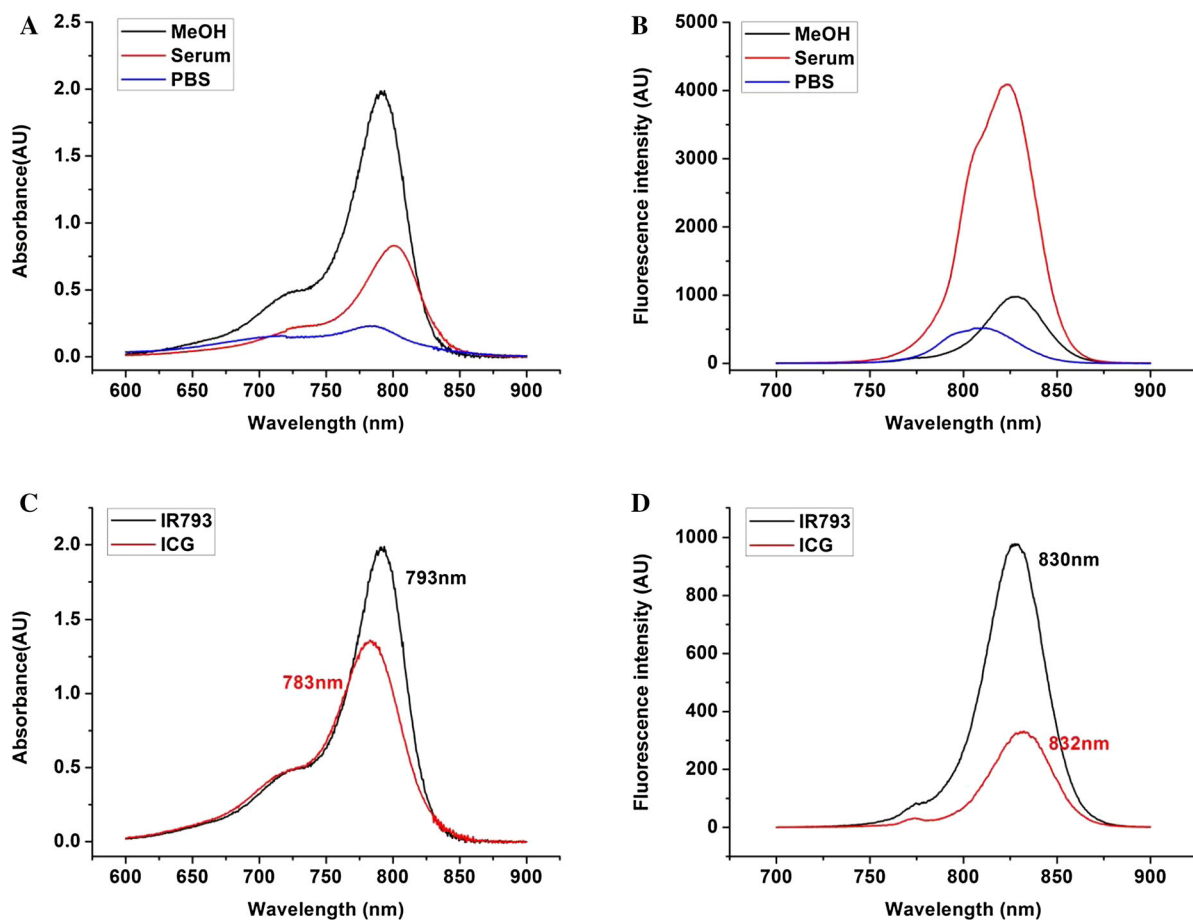
### Confocal imaging in living cells

A549 cells were grown on glass-bottom culture dishes. After incubation with IR-793 (10  $\mu\text{M}$ ) and ICG (10  $\mu\text{M}$ ) in FBS-free 1640 medium at 37 °C for 30 min and washing thrice with PBS buffer (pH 7.4), the cells were immediately imaged using a confocal microscope with an objective lens ( $\times 20$ ). Excitation was at 793 nm when the cells were incubated with IR-793 and 805 nm when the cells were incubated with ICG.

## Results and discussion

### Characterization of IR-793

IR-793 was synthesized using a simple method (Scheme 1). The structure of IR-793 was confirmed via <sup>1</sup>H-NMR and MS. <sup>1</sup>H-NMR (400 MHz, CD<sub>3</sub>OD):  $\delta$ (ppm) = 1.78 (s, 14H), 2.32 (s, 6H), 2.53–2.56 (t, J = 6 Hz, 4H), 5.40 (s, 4H), 6.32–6.35 (d, J = 14 Hz, 2H), 7.16–7.22 (m, 8H), 7.35–7.37 (d, J = 8.4 Hz, 2H), 7.87–7.89 (d, J = 8.4 Hz, 2H), 7.97 (s, 1H), 8.39–8.43 (d, J = 14.4 Hz); time-of-flight MS m/z: 822.2[M] (<sup>1</sup>H-NMR and MS spectra of IR-793, as shown in Supplementary Fig. 1) confirmed the formation of IR-793.



**Fig. 1** The absorption and fluorescence spectral property of IR-793 were examined in PBS, methanol solutions or serum. **a** and **b** The absorption and emission spectra of IR-793 (10  $\mu$ M) in

methanol (MeOH), PBS, and serum. **c** and **d** The absorption and emission spectra of IR-793 (10  $\mu$ M) and ICG (10  $\mu$ M) in methanol

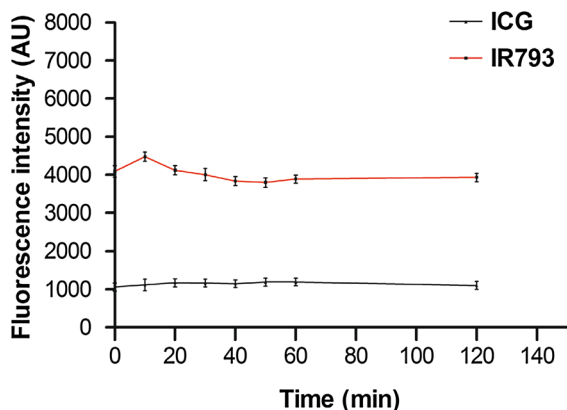
### Optical properties of IR-793

We detected the absorption and emission spectrum of IR-793 in methanol, serum, and PBS (Fig. 1a, b). The maximal absorption and emission wavelength of IR-793 in serum showed a red shift compared with that in methanol and PBS, as shown in Fig. 1a. The emission intensity was considerably higher in serum than that in methanol and PBS, as shown in Fig. 1b. The absorption and emission peak of IR-793 in different media (methanol, serum, and PBS) were in the NIR region (600–900 nm). The excitation and emission spectra of IR-793 were located at 793 and 830 nm in methanol, 779 and 810 nm in PBS, and 799 and 823 nm in serum, which emitted a similar emission peak in methanol to Indocyanine Green (ICG), as shown in

Fig. 1c, d. Moreover, the total fluorescence intensities of IR-793 and ICG in the serum were maintained at a stable level for 120 min, which indicated that IR-793 was stable similar to ICG in the serum (Fig. 2). However, the fluorescence intensity of IR-793 in the serum was higher than that of the ICG at the same concentration. These results suggested that IR793 had promising potential as a NIR probe in biomedical imaging.

### Cytotoxicity of IR-793

When using probes in living cells, their biocompatibility is always the first property to consider (Funayama et al. 2012). Therefore, cytotoxicity was measured via MTT assay to quantify the living A549



**Fig. 2** Fluorescence stabilities of IR-793 and ICG (10  $\mu$ M) were evaluated in 100 % FBS at 37 °C for 120 min. Each valid point and vertical bar shows mean  $\pm$  SD (n = 3)

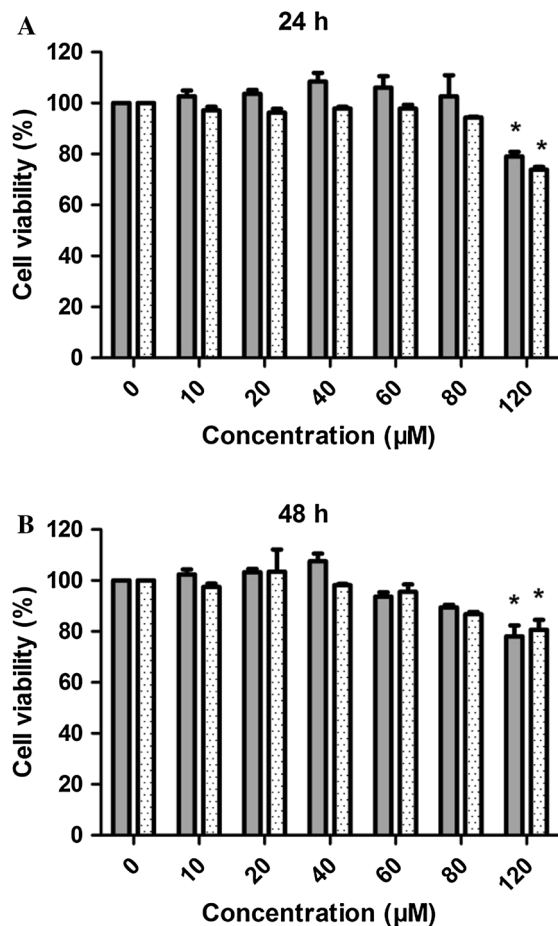
cells in the wells after incubation with various concentrations of IR-793. The results indicated that the cell viability of A549 cells was not altered by IR-793 unlike with ICG, even in the wells with the highest probe concentration (120 nm). The bar graph in Fig. 3 indicated cell viability to be above 75 % at the maximum concentration of 120 nm after 24 and 48 h of incubation. Cytotoxicity assay proved that IR-793 had no apparent toxicity to living cells. These results proved that IR-793 probe was safe in living cells.

#### In vitro cell imaging

A confocal microscopy analysis was performed to study the applicability of IR-793 in biological systems compared with ICG. A549 cells were inoculated in IR-793 at a concentration of 10  $\mu$ M with good cell viability for 30 min. The distribution of the probe within the cells was observed under a fluorescence microscope following excitation at 793 nm. Figure 4 showed that IR-793 and ICG can permeate into the membrane, and the fluorescence intensities varied within single cells. The studies showed that IR-793 demonstrated excellent membrane permeability and good photostability.

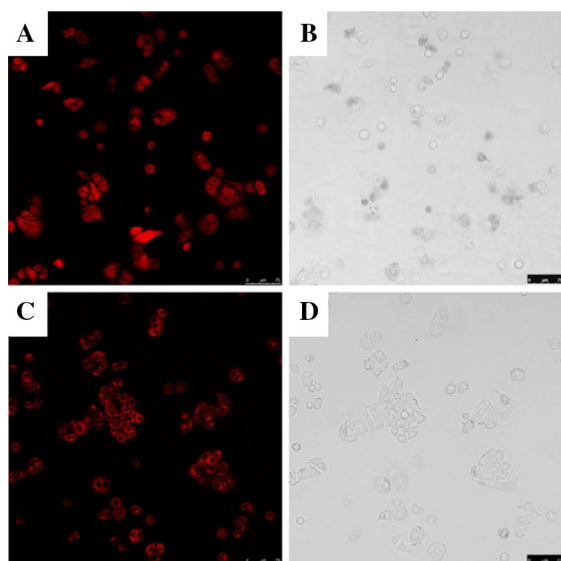
#### Conclusion

The need to develop new, reliable, and low-cost NIR fluorescence probes for cell imaging and disease diagnosis is increasing. In the present study, we



**Fig. 3** The viability of A549 cells cultured with IR-793 in comparison with ICG at the same dose incubated for 24 and 48 h. Solid bars represent ICG, while dotted bars represent IR-793. Data were presented as the mean cell viability with standard deviation after triplicate analysis. *P* value < 0.05 is indicated by one asterisk

developed a modified procedure for the chemical synthesis of a highly fluorescent, novel NIR fluorescence probe (IR-793) to improve the overall yield for potential large-scale preparation. IR-793 exhibited better water solubility and optical stability in serum than ICG when applied in cellular analysis in an aqueous environment. The excellent water solubility of IR-793 ensured its benefit for in vivo imaging and its biocompatibility with cells (Amiot et al. 2008). IR-793 demonstrated outstanding optical properties and photostability in serum, which allowed living cells with minimal NIR background fluorescence from the hosts to achieve excellent signal-to-background ratio. Furthermore, IR793 had low cytotoxicity to living



**Fig. 4** Confocal fluorescence images of living cells, A549 cells. **a** Confocal fluorescence images of living cells A549 incubated with 10  $\mu$ M IR-793 at 37  $^{\circ}$ C for 30 min, **b** bright field image of living A549 cells, **c** confocal fluorescence images of living cells A549 incubated with 10  $\mu$ M ICG at 37  $^{\circ}$ C for 30 min, and **d** bright field image of living A549 cells

A549 cells. In conclusion, the aforementioned preliminary evaluation results indicated that IR-793 has higher prospects as a biocompatible NIR fluorescent sensor for sensitive intracellular imaging compared with ICG. This simple and sensitive NIR fluorescent probe will be considerably useful to biomedical researchers on biological systems, such as in the real-time monitoring of cells and image-guided oncologic surgery, as a fluorescent probe with chemically conjugated specific ligands. In our future work, we will focus on systematic investigations of photo-dependent cytotoxic activities to broaden the application of IR-793.

**Acknowledgments** This work is supported by National High-tech Research and Development Project (863 Project, 2013AA032205), Industry Project of Jiangsu Science-technology Support Plan (BE2013840), Science and Technology Development Program of Suzhou (ZXY201412), the Graduate Research and Innovation Program of Jiangsu Province in China (KYLX15-0170).

**Supporting information** Supplementary Figure— $^1$ H-NMR and MS of IR-793.

## References

- Amiot CL, Xu S, Liang S, Pan L, Zhao JX (2008) Near-infrared fluorescent materials for sensing of biological targets. *Sensors* 8:3082–3105
- Chang SK, Rizvi I, Solban N, Hasan T (2008) In vivo optical molecular imaging of vascular endothelial growth factor for monitoring cancer treatment. *Clin Cancer Res* 14(13):4146–4153
- Funayama T, Sakane M, Abe T, Ochiai N (2012) Photodynamic therapy with indocyanine green injection and near-infrared light irradiation has phototoxic effects and delays paralysis in spinal metastasis. *Photomed Laser Surg* 30:47–53
- Luo S, Zhang E, Su Y, Cheng T, Shi C (2011) A review of nir dyes in cancer targeting and imaging. *Biomaterials* 32:7127–7138
- Mamoon AM, Gamal-Eldeen AM, Ruppel ME, Smith RJ, Tsang T, Miller LM (2009) In vitro efficiency and mechanistic role of indocyanine green as photodynamic therapy agent for human melanoma. *Photodiagnosis Photodyn Ther* 6:105–116
- Mather S (2009) Molecular imaging with bioconjugates in mouse models of cancer. *Bioconj Chem* 20:631–643
- Nakayama A, Bianco AC, Zhang CY, Lowell BB, Frangioni JV (2003) Quantitation of brown adipose tissue perfusion in transgenic mice using near-infrared fluorescence imaging. *Mol Imaging* 2:37–49
- Rasmussen JC, Tan IC, Marshall MV, Fife CE, Sevick-Muraca EM (2009) Lymphatic imaging in humans with near-infrared fluorescence. *Curr Opin Biotechnol* 20:74–82
- Sevick-Muraca EM (2012) Translation of near-infrared fluorescence imaging technologies: emerging clinical applications. *Annu Rev Med* 63:217–231
- Sun C, Wang P, Li L, Zhou G, Zong X, Hu B, Zhang R, Cai J, Chen J, Ji M (2013) A new near-infrared neutral PH fluorescent probe for monitoring minor PH changes and its application in imaging of hepg2 cells. *Appl Biochem Biotechnol* 172:1036–1044
- Sun C, Wu Y, Cai J, Wang P, Zong X, Zhou G, Li L, Ji M (2014) Synthesis of a near-infrared fluorescent probe and its application in imaging of mcf-7 cells. *Biotechnol Lett* 36:1203–1207
- Sun C, Cai J, Chen J, Wu Y, Wang P, Zhou G, Zong X, Chen B, Lv Y, Ji M (2015) The synthesis of a novel near-infrared fluorescent probe and its application in imaging of living cells. *Appl Biochem Biotechnol* 175:1644–1650
- Xu H, Baidoo K, Gunn AJ, Boswell CA, Milenic DE, Choyke PL, Brechbiel MW (2007) Design, synthesis, and characterization of a dual modality positron emission tomography and fluorescence imaging agent for monoclonal antibody tumor-targeted imaging. *J Med Chem* 50:4759–4765
- Zhang C, Liu T, Su Y, Luo S, Zhu Y, Tan X, Fan S, Zhang L, Zhou Y, Cheng T, Shi C (2010) A near-infrared fluorescent heptamethine indocyanine dye with preferential tumor accumulation for in vivo imaging. *Biomaterials* 31:6612–6617