A New Method based on Peptide Mediated for Intracellular Linc-ROR Detection

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Research Article

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Abstract

Linc-ROR, as a new intergenic lncRNA, over-expressed in many kinds of cancer that promotes cancer cell proliferation. So, there are significant values of Linc-ROR situ detectiction in diagnosis and research of diseases. While, the methods for LncRNA detection are almost outside the cells. In this study, established a method for Linc-ROR detection inside the cells. First, we put the nucleic acid probe and antibody together, probe entered cell by peptide (L17E) mediated endocytosis; In order to decreased background signals, the quenching probes were deliveried into cells to neutralise free fluorescence probes. We verified the feasibility of this method, it has a huge amount of potential in clinical test.

1. Introduction

Long non-coding RNAs (lncRNAs) are a type of RNA, defined as being transcripts with lengths exceeding 200nt that are not translated into protein[1,2]. Linc-ROR, as a new intergenic lncRNA, over-expressed in many kinds of cancer that promotes cancer cell proliferation, including breast cancer (BC), pancreatic cancer (PC), colorectal cancer (CRC) and so forth. As linc-ROR can regulate cell proliferation, apoptosis, migration, and invasion, it can thus be used as a potential biomarker for patients with tumors and has potential clinical significance as a therapeutic target[3–5].

At present, methods for the detection of RNAs include polymerase chain reaction (PCR), RNA-seq, gene chip or northern blotting[6–10]. Although these methods do have their own advantages, but some drawbacks limit their use in clinical detection. Firstly, these methods involve extracting RNAs from cells, while linc-ROR are not stable and easily degraded by Ribonuclease R (RNase R)[11]. Then, current methods can only obtain expression quantity of lncRNAs, but ignore the position of lncRNAs in different cells[12.13]. Therefore, development of rapid, simple, sensitive diagnostic systems for intracellular linc-ROR detection is emerging as a necessary response to develop of clinical laboratory diagnosis and life science research.

There are several difficulties about intracellular linc-ROR detection. At first, we need deliver detection fluorescence probe (FP) to cells. While, nucleic acid FPs are negatively charged molecules, they cannot diffuse across cell membranes[14]. The FP deliver of traditional methods are assisted by liposome or nanomaterial[15–17]. Liposome is an artificial vesicle composed of one or more concentric phospholipid bilayers and used especially to deliver microscopic substances to body cells[18]. In previous study, we found that although liposome could deliver FPs into cells, while the cytotoxicity and the transfer efficiency may warrant limiting its usefulness[19]. FPs mediation methods based on nanomaterials have developed rapidly in recent years because of their dual functions of quenching fluorescence and carrying FPs[20,21]. However, nanomaterials are expensive, complicated, time-consuming and persistent cytotoxicity[22]. Our previous study found that FPs are closely bound to nanomaterials with low release efficiency, more than 40μg/mL of graphene oxide (GO) was needed to fully quench the 100nM fluorescent-modified single-stranded nucleic acid probe. Because of lncRNA are much longer than micRNA, these methods are not suitable for lncRNA detection. they are mainly using for detection of

micRNA[20,21]. Endocytosis is a cellular process in which substances are brought into the cell. The material to be internalized is surrounded by an area of cell membrane, which then buds off inside the cell to form a vesicle containing the ingested material. It is a form of active transport[23,24]. So endocytosis is a simple and rapid process without any damage to cells, which is an ideal way of FPs delivery. Antibodies and other biological macromolecules can enter cells through endocytosis easily. In this study, we combined FP with antibody and introduce it into cell through endocytosis.

While, one of the main barriers of this method is the limited release of FPAs from endosomes into the cytosol. FPAs are taken up by cells via endocytoses, which is a process that involves the physiological uptake of extracellular substances delivered into cells by encapsulation into vesicular compartments named endosomes. Without their release from vesicular compartments into the cytosol, FPAs cannot reaction with their target molecules, and may finally be degraded in these compartments[25,26]. Misao Akishiba and his co-workers reported a new lipid-sensitive endosomolytic peptide named L17E, L17E had efficient endosomolytic activity that achieve a similar extent of endosomolytic activity with less of the peptide[27]. So, we used lipid-sensitive polypeptide L17E to deliver FPAs in to cells.

In addition, uncombined FPAs are able to increase background signals without restrictive measures. So, one kind of quenching probe (QP) was designed to combine free FPA and reduce the background signal. The QP was modified with a secondary antibody (QPSA) which could bind to the antibody on the FP. After the QPs were delivered into the cells with the same manner, the antibody on the FPs and the secondary antibody on the QPs would band together. If the FPAs were free, they would rapidly hybridize with the QPSAs through the proximity ligation effect[28–30] and the fluorescent signal would be quenched. Therefore, we can get the concentration of the target according to the fluorescence intensity.

2. Experimental

2.1 Cell culture

Human breast cancer cells (MDA-MB-231, BT549 and HAC15) were obtained from American Type Culture Collection (ATCC). Cells were maintained in DMEM/F-12 medium (C11330500BT, Gibco) with 10% FBS (Gibco, 10099141), 1% PS (HyClone, SV30010) at 37°C in a tri-gas incubator containing 5% CO2 under normoxic (21% O2) condition. The genotyping of these breast cancer cells shared > 94% of the STR loci with the ATCC original clones by STR identification (Genewiz, Suzhou).

2.2 Extracellular Fluorescence detection

All oligonucleotide sequences and polypeptide were synthesized and purchased from Sangon Biotechnology Co. (Shanghai, China), and the sequences were provided in Table S1. Fluorescence signals were recorded using a Cary Eclipse Fluorescence spectrophotometer (Agilent, California).

Parameters of scan: Scanning intervals, 1 nm; scanning speed, 600 nm/min; Excitation slit, 10 nm; Emission slit, 10 nm.

2.3 Gel electrophoresis

The 10% BeyoGel™ Plus Precast PAGE Gel was purchased from Beyotime (Shanghai, China), and it was carried out at a constant voltage at 100V for 1h. Electrophoresis apparatus (DYY-6C, LIUYI, China) was used for the electrophoresis experiments. The gel was photographed by Bio-RAD digital imaging system.

2.4 Cellular uptake of FPAs and QPSAs

Breast cancer cells MDA-MB-231 were seeded into 96 well cell culture plate (NEST), and the cells were allowed to reach 80–90% confluence in 24h. These cells were washed twice with serum-free medium, and then incubated with 200nM probes with or without the presence of a peptide (10 μM) in serum-free medium for 2h at 37°C. Cells were washed with serum-free medium and incubated in serum-supplemented medium for 2 h at 37°C. The cellular uptake of probes was analysed in live cells using Axio Observer A1 inverted fluorescence microscope.

3. Results And Discussion

3.1 Synthesis of antibody-DNA Conjugates

1 μL of a 4 mM solution of sulfosuccini-midyl 4-(N-maleimidomethyl)-cyclohexane-1-carboxylate (sulfo-SMCC) in anhydrous DMSO was added 10 μL of the mouse monoclonal antibody FBXO2 (33kDa) solution (200 μg/mL) and incubated at RT for 2h. Then added 500 μL reaction buffer (PBS 1X, 20 mM EDTA, pH 7.2) and centrifuged for 15 min at 14000g in 3k MWCO centrifugal filter column twice. Free sulfo-SMCC was separated from the solution to a ultima volume of 60 μL. Thiolated single strand DNA was suspended to 100 μM in reaction buffer. 3 μL of the 100 μM DNA was added to 50 μL of reaction buffer. Then, four μL of a 100 mM solution of dithiothreitol was added to decrease the thiolated DNA. After that, the solution was incubated at 37°C for one hour. Then, 7k MWCO gel microspin columns (Life Technologies) were equilibrated with reaction buffer. The reduced oligonucleotides were then desalted by the equilibrated microspin columns twice. The solutions were mixed and l reacted 12 h at 4°C, and then purified by 10k MWCO filter column[31,32]. The combination of antibody and DNA probes were determined by polyacrylamide gel electrophoresis (PAGE) analysis. The signal came from FAM labeled on DNA probes. As shown in Fig. 1A, lane 1 is FP only and lane 2 is a result of the addition of FP to antibody. The FP and antibody form a complex and move more slowly.

3.2. Extracellular validation

Ideally, quenching probes (QPSAs) only react with free FPAs when enter into cells (Fig. 1B top). As shown in Fig. 1C, there is a strong fluorescent signal when we added 200 nM FPAs to 100 nM linc-ROR targets (DFTs). Then, the fluorescent signal among fell by close to 50 percent after superfluous QP1 joined. The result proved that free FPAs were quenched. While, the fluorescent signal had decreased dramatically after change QP1 to QPSA1. This suggested that when the bases were completely complementary, FPA/DFT would be competitive displaced by QPSA1 for more strong binding force (Fig. 1B middle). In

order to solve this problem, we try to reduce the complementary base pairs between FPA and QPSA. The experiment results shown that QPSA3 with sixteen pairs of complementary bases can reach a balanced change (Fig. 1B bottom). So, we chose QPSA3 as the optimum condition for the following research.

3.3. Intracellular validation

The strategy is illustrated in Fig. 2A. The lytic activity of L17E was weaker, because of a negatively charged Glu residue was inserted in the hydrophobic face. The net positive charges of the peptide can help its endocytosis uptake and cell surface adsorption (Figure S1). FPAs were delivered into the cells through L17Es mediated endocytosis. Then, the peptide preferentially perturbs the endosomal membranes to attain the cytosolic release of FPAs with sufficient efficacy to Htarget detection.

Next, we evaluated the ability of these peptides to promote the take in and cytosolic release of endocytosed biomacromolecules. All the results shown in Fig. 2B. MDA-MB-231 cells were incubated with probes and L17Es in medium for two hours, and the cellular distribution of the fluorescence signal was analysed using Axio Observer A1 inverted fluorescence microscope. On the other hand, when cells were treated with FPAs in the presence of L17E (10 μM), a significant expression of fluorescence signal was observed in the cells (Fig. 2B bottom), which suggests that the L17E-mediated delivery of FPAs into cells. In contrast, no significant fluorescence signals were observed after treatment with FPAs in the absence of L17E (Fig. 2B top). In addition, the experimental results shown that only nucleic acid probes catenating with antibodies can be delivered to cells. These results collectively indicate that L17E allows the delivery of FPAs into the cytosol.

3.4 Cytosolic delivery of QPSA using L17E

Free FPAs without hybridization with linc-ROR targets were background signal. In this study, we innovation used a kind of quenching probe which bound with anti-mouse secondary antibody (QPSA) to decrease background signal. The QPSAs were labeled with Dabcyl which could quench the fluorescence of FAM when they were closed. The QPs and secondary antibodies were forming QPSAs at the same way. Then, the QPSAs were delivered into the cells with the same method (Fig. 3A). Applying the principle of specific binding of antibody and secondary antibody, the FPs and QPs were closed through proximity ligation assay and the fluorescence were quenched. While, antibody-DNA probes hybridization with linc-ROR would not be quenched and keep a strong fluorescence.

compared to the control group (Fig. 3B top), the fluorescence signal could not change when QPSAs incubated with cells in the absence of L17Es (Fig. 3B middle). While, the fluorescence signal significant declined in the absence of L17Es (Fig. 3B bottom). All results verified that QPSAs could be delivered to cells and free FPAs would be quenched.

3.5 Verify in other tumor cell lines

Then, the proposed strategy was used to estimate the intracellular expression levels of Linc-ROR in different living cancer cells, breast cancer cell BT-549 and adrenocortical cell HAC15. As shown in Fig. 4, as for MDA-MB-231 and BT-549 cells, two breast tumor cell lines, we could observe bright FPA

fluorescence images. Also, the fluorescence intensity of FPA in BT-549 cells was stronger than MDA-MB-231 cells. While, the fluorescence intensity of HAC15 cells was very week. The results were in keeping with Linc-ROR RNA Q-PCR results (Figsure S2).

Conclusions

In this report, we have demonstrated a new detection strategy for Linc-ROR in cells. First, we linked the nucleic acid probe and antibody together. Then, L17E a single negatively charged Glu residue into the potentially hydrophobic face of M-lycotoxin was used to delivery probes into cells through endocytosis. In order to decreased background signals, the quenching probes were deliveried into cells at the same way. At last, we verify the feasibility of this method, it has a huge amount of potential in clinical test. Furthermore, our approach can also be potentially applied for detect of other RNAs in cells.

Declarations

Notes

The authors declare no competing financial interest.

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Figures

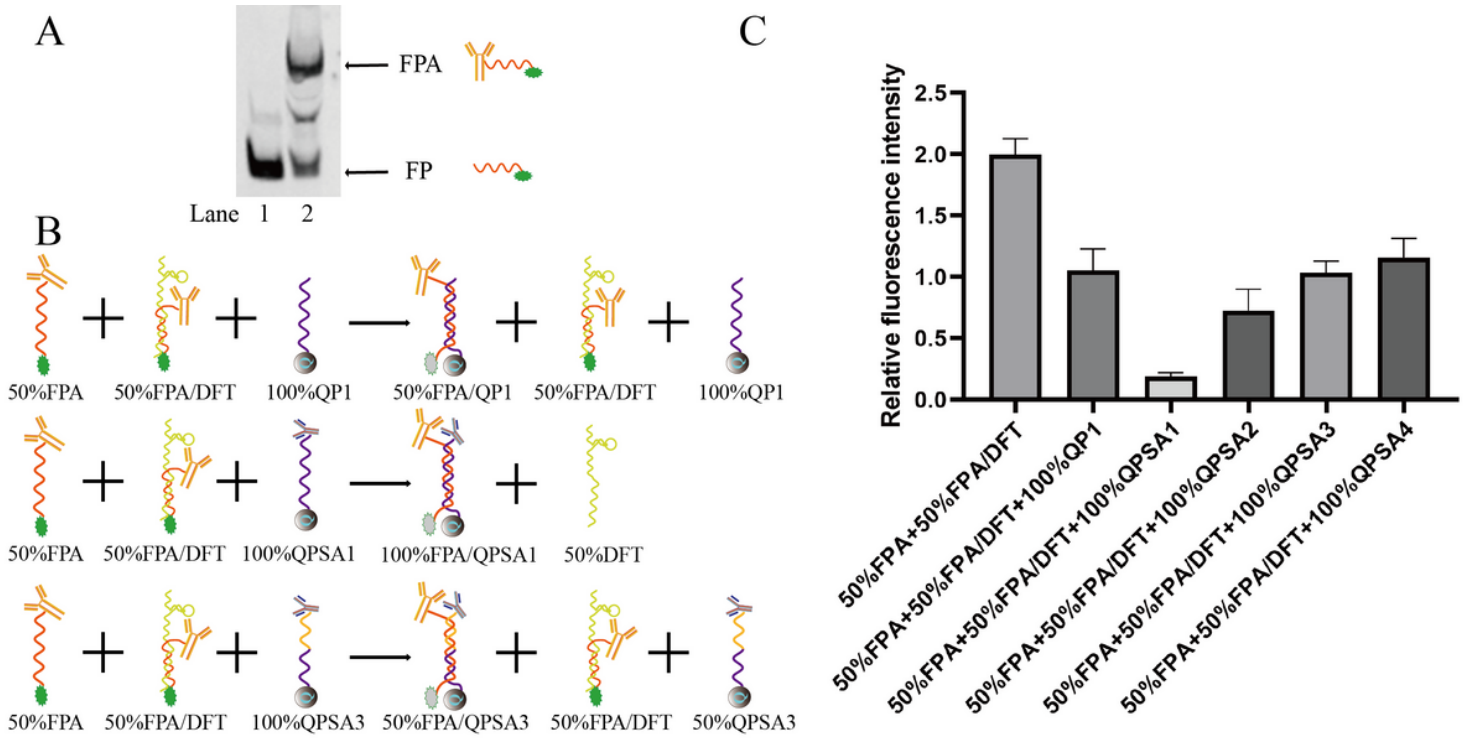

Figure 1

(A) Samples were resolved by PAGE. Lane 1 is unmodified FP, lane 2 is FP-antibody conjugate. A significant mass shift was observed in lanes due to the addition FP to the antibody. The signals came from FAM labeled on FP. (B top) Ideally, quenching probes (QPSAs) only react with free FPAs. (B middle) when the bases were completely complementary, FPA would be competitive displaced by QPSA1. (B bottom) when 16 bases complementary, FPA would not be displaced by QPSA3. (C) 50%FPA+50%FPA/DFT: 200 nM FPA react with 100 nM DFT; 50%FPA+50%FPA/DFT + 100% QP1: 200 nM FPA + 100 nM DFT react with 200 nM QP1, 40 bases complementary; 50%FPA+50%FPA/DFT + 100% QPSA1: 200 nM FPA + 100 nM DFT react with 200 nM QPSA1, 40 bases complementary; 50%FPA+50%FPA/DFT + 100% QPSA2: 200 nM FPA + 100 nM DFT react with 200 nM QPSA2, 20 bases complementary; 50%FPA+50%FPA/DFT+100%QPSA3: 200 nM FPA + 100 nM DFT react with 200 nM QPSA3, 16 bases complementary; 50%FPA+50%FPA/DFT+100%QPSA4: 200 nM FPA + 100 nM DFT react with 200 nM QPSA4, 12 bases complementary.

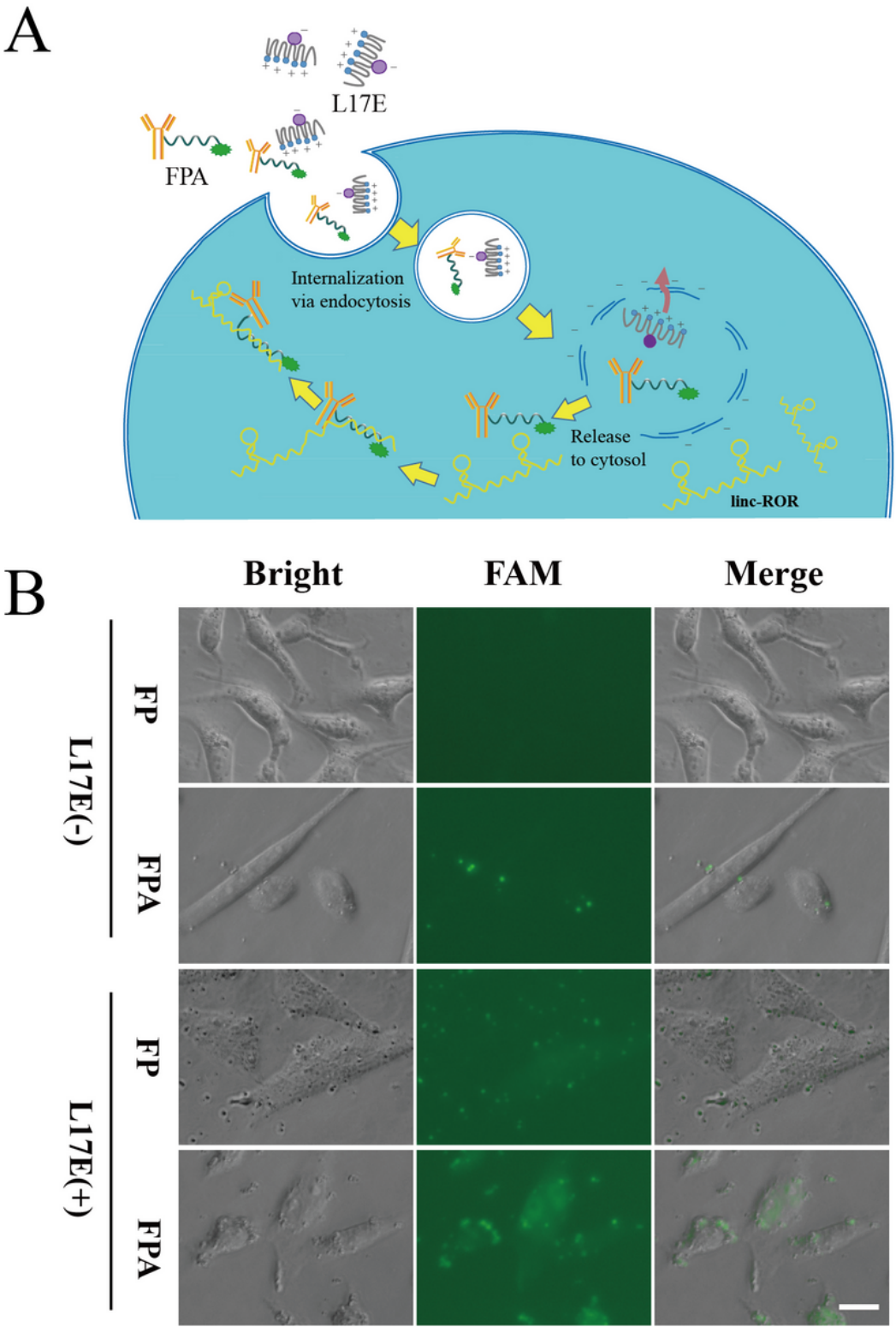

Figure 2

(A) The principle of peptide L17E mediate detection probes enter the cells. (B top) Fluorescence images of MDA-MB-231 cells incubated with FP or FPA (200 nM) for 2h in the absence of L17E. (B bottom) Fluorescence images of MDA-MB-231 cells incubated with FP and FPA (200 nM) for 2h in the presence of L17E (10 μM). Scale bar, 20 μm.

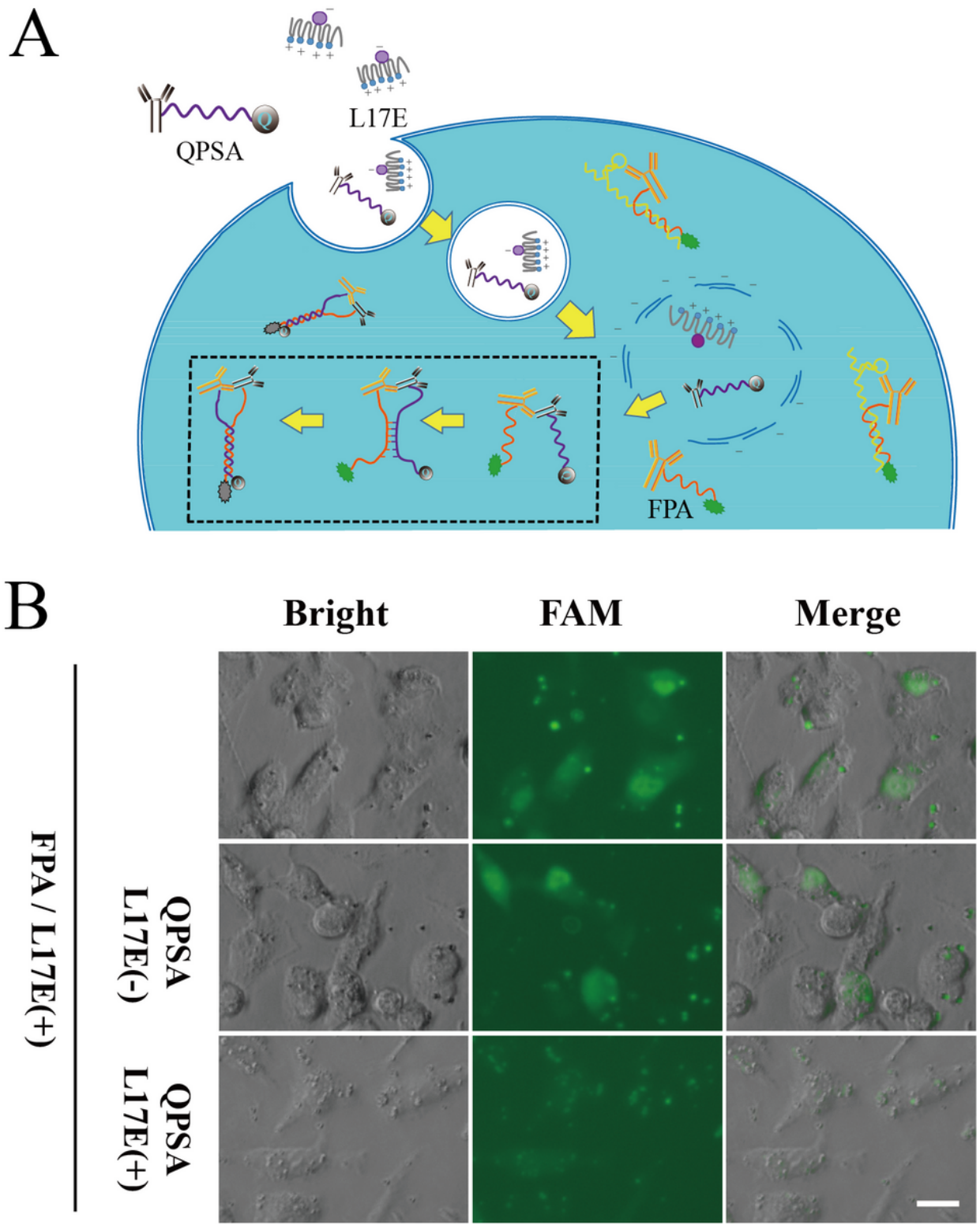

Figure 3

(A) The principle of peptide L17E mediate quenching probes enter the cells. (B top) Fluorescence images of MDA-MB-231 cells incubated with FPA (200 nM) for 2h in the absence of L17E. (B middle) Fluorescence images of MDA-MB-231 cells incubated with FPA (200 nM) for 2h in the presence of L17E (10 μM), then incubated with QPSA (200 nM) for 2h in the absence of L17E (10 μM). (B bottom)

Fluorescence images of MDA-MB-231 cells incubated with FPA (200 nM) for 2h in the presence of L17E (10 μM), then incubated with QPSA (200 nM) for 2h in the presence of L17E (10 μM). Scale bar, 20 μm.

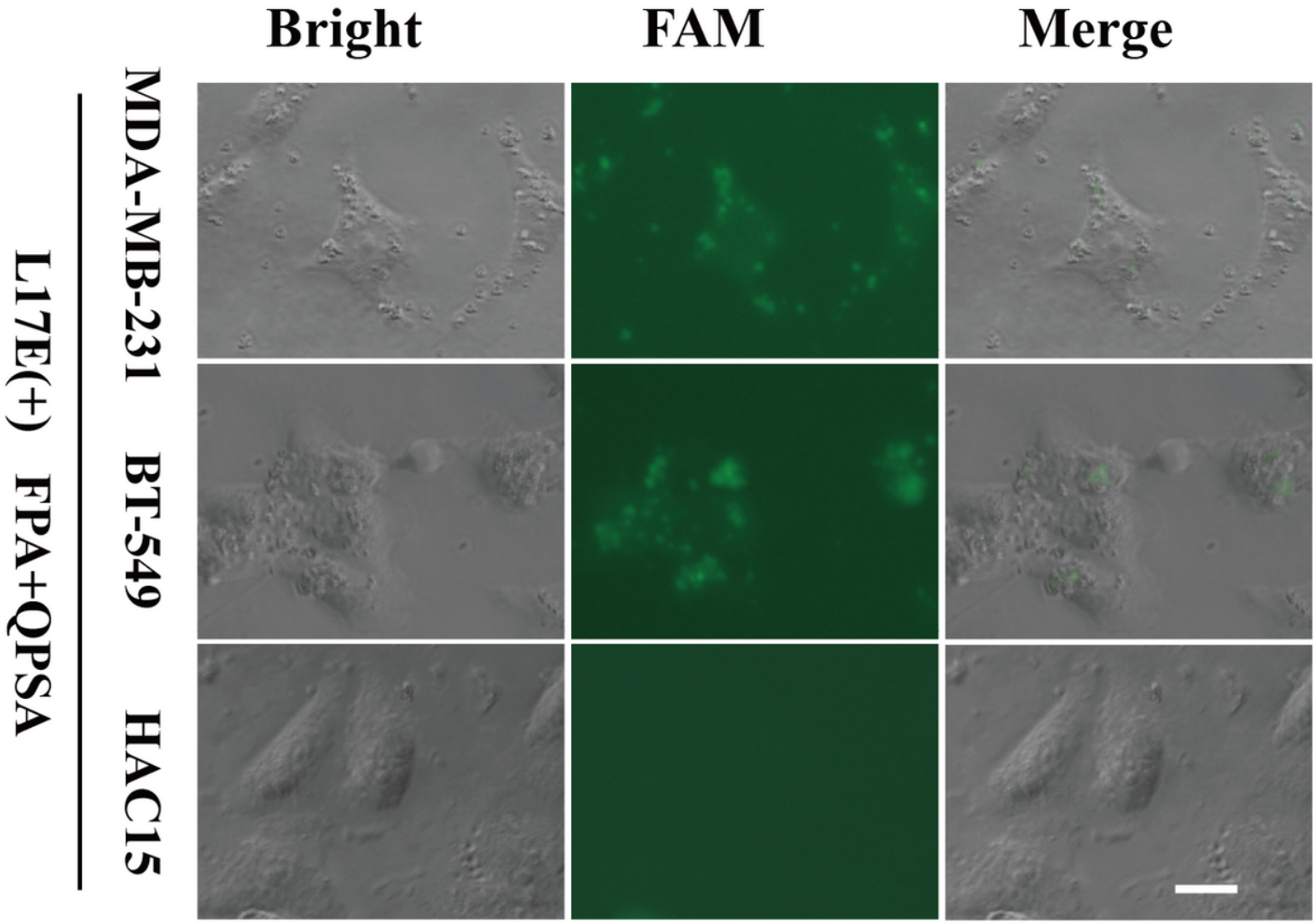

Figure 4

Fluorescence images of MDA-MB-231, BT-549 and HAC15 cells. First, these cells incubated with FPA (200 nM) for 2h in the presence of L17E. Then, these cells incubated with QPSA (200 nM) for 2h in the presence of L17E. Scale bar, 20 μm.

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