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IR-780 Dye for Near-Infrared Fluorescence Imaging in Prostate Cancer

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Background: The aim of this study was to investigate near-infrared fluorescence (NIRF) imaging as a novel imaging modality that allows for early detection of cancer and real-time monitoring to acquire related information. IR-780 iodide, a lipophilic dye, accumulates selectively in breast cancer cells and drug-resistant human lung cancer cells, with a peak emission at 780 nm that can be easily detected by the NIRF imaging system. The application of IR-780 for prostate cancer imaging was thoroughly investigated to further expand its clinical value.

Material/Methods: The impact of IR-780 on the survival of prostate cancer cells PC-3 and LNCaP as well as normal prostate epithelial cells RWPE-1 was determined. Duration of IR-780 dye staining was optimized in PC-3 cells. The involvement of specific OATP1B3 inhibitor in the selective accumulation of IR-780 was investigated. IR-780 for prostate cancer imaging was carried out in athymic nude mouse models and, acute toxicity of IR-780 was evaluated.

Results: IR-780 incubation resulted in a dose-dependent inhibition to cell proliferation. Mean fluorescence intensity of prostate cancer cells peaked at 20-min IR-780 incubation. Specific uptake of IR-780 dye in prostate cancer cells was mainly through the function of OATP1B3. We also demonstrated that NIRF dye effectively identified the subcutaneous prostate cancer xenografts, subsequently confirmed by histological examination. There was no significant impact on the physical activity, weight, and tissue histology of BABL/C mice with 10-fold imaging dose of 1-month IR-780 dye administration.

Conclusions: NIRF imaging using IR-780 dye is a feasible and practicable method for prostate cancer detection, with potential tumor-killing ability, although more investigations are needed before clinical translation.

MeSH Keywords: Fluorescent Dyes • Optical Imaging • Organic Anion Transporters • Prostatic Neoplasms • Spectroscopy, Near-Infrared

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Background

Prostate cancer (CaP) is the leading cancer type in men, accounting for 27% of cancer incidence according to the data provided by American Cancer Society in 2014 [1]. Most patients with CaP at an advanced stage cannot avoid the occurrence of life-threatening deterioration and metastasis despite application of multiple therapies [2]. Various imaging modalities, including radiology and ultrasonography, provide extensive information for early cancer detection in current clinical practice. However, there still exist unsolved issues such as portability for quick scanning, the sensitivity in detecting minimal lesions or metastases, and safety concerns about radiation [3]. Therefore, novel cancer-specific molecular imaging techniques are still urgently needed.

Near-infrared fluorescence (NIRF) imaging as a novel imaging modality has attracted extensive attention in the last 2 decades. This technique allows for detection of various pathophysiological states in cells as well as real-time monitoring to acquire related information [4]. To this purpose, specific NIRF probes with emission wavelength in the near-infrared window are administered in advance. Polymethine cyanine dyes such as indocyanine green (ICG) have been applied in clinical and experimental settings, but the lack of specificity limits their uses. Target-specific probes display unique properties of tunable optical characteristics and targeting ability obtained through bio-conjugations of non-targeting NIR fluorophores and active metabolic substrates or antibodies as the targeting moiety [5].

IR-780 iodide is a lipophilic dye with a peak emission at 780 nm and can be easily detected by the NIRF imaging system. It is reported that IR-780 can accumulate selectively in breast cancer cells and drug-resistant human lung cancer cells [6]. Although the function of organic anion transporting peptides (OATP) has been proposed to explain the specific accumulation, the underlying mechanism remains elusive [7]. OATPs are 12-transmembrane glycoproteins originated from the SLCO gene superfamily [8], which are constitutively expressed in several epithelial tissues throughout the body. OATPs overexpression also affects cancer development, including OATP1B3, in prostate cancer [9,10]. To further expand their clinical value in prostate cancer detection, we first determine the impact of IR-780 on the survival of prostate cancer cells and normal prostate epithelial cells. Optimized IR-780 dye-mediated imaging was explored through *in vivo* and *in vitro* studies. The underlying mechanism of IR-780 accumulation was also investigated by specific OATP1B3 inhibitor. The results suggest that specific uptake of these organic dyes in various prostate cancer cells is mainly through the function of OATP1B3. We also demonstrated that NIRF dye-mediated imaging is a feasible and practicable method for prostate cancer detection.

Material and Methods

Chemical reagents

Cyanine dyes IR-780 iodide were purchased from Sigma-Aldrich (St. Louis, MO), prepared as stock solutions (1 mM) in dimethyl sulfoxide (DMSO, Sigma-Aldrich) and stored at 4°C in the dark. Working solutions were made from IR-780 dye diluted in serum-free media and filtered through 0.2-μm filters before use.

Cell lines and cell culture

Human prostate cancer cell lines PC-3, LNCaP and normal prostate epithelial cell line RWPE-1 were purchased from the American Type Culture Collection (ATCC) and grown according to ATCC recommendations. Each type of recommended medium (RPMI-1640 for LNCaP and RWPE-1, F-12K for PC-3, Invitrogen) contains 10% fetal bovine serum (Gibco) and penicillin (100 IU/mL)/streptomycin (100 μg/mL) in a humidified atmosphere with 5% CO_2 at 37°C.

Viability assay

For the cell viability assay, cells were exposed to 0, 2.5, 5, 10, 20, 40, 80, and 160 μM of IR-780 dye and then assessed for the inhibition of cell proliferation after 24 h exposure using the CCK-8 assay kit (Beyotime, Beijing, China). In brief, 100 μL PC-3, LNCaP, and RWPE-1 cells were seeded in 96-well plates (5×10^3 cells/well) and continued to grow 12 h for cell adhesion. After 24-h incubation with IR-780, cells were washed 2 times using 100 μL of fresh medium. Then, 10 μL of CCK-8 reagent was added into each well and incubated at 37°C for 4 h. Finally, the absorbance in each well was read using an xMark™ microplate absorbance spectrophotometer at 450 nm (Bio-rad, Hercules, CA, USA). Cell viability at different IR-780 concentrations was described as percentages of OD value compared to that in the control group without dye addition.

Staining optimization of IR-780 dye

PC-3 cells were incubated with 20 μM IR-780 dye for 5, 10, 15, 20, 30, 40, 50, and 60 min to define the optimal time for cell staining. We placed 1×10^4 PC-3 cells were placed into 35-mm glass-bottom petri dishes (NEST, Shanghai, China) that was designed for confocal laser scanning microscopic observation and continued to grow them for 24 h. After the removal of culture medium, working solutions of IR-780 dyes (20 μM) were added. Cells were washed twice at different time points, then observed and imaged under laser confocal microscopy with fixed imaging parameters (OLYMPUS FV1000, Shinjuku-ku, Tokyo, Japan). Mean fluorescence intensity (MFI, minimum to maximum, 0 to 255) was analyzed by Image pro plus software 6.0,

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which was calculated from the images captured under ten 10 random visual fields (×200) in each group.

Migration/invasion assay

Cultured PC-3 and LNCaP cells were re-suspended in serum-free medium (5×10^4 cells/ml). We placed 200 µL cells into the upper chambers, then added 1% FBS and 10% FBS to the upper and the lower chamber, respectively. For invasion assay, matrigel (100 µg/mL) was coated into the upper chamber before cell seeding. After incubation for 24 h, cells in the upper chamber were gently swiped out. The cells that migrated/invaded to the lower chamber were fixed with 4% paraformaldehyde for 10 min and stained with DAPI, as previously reported [11]. The number of fixed cells with blue staining was counted under an Olympus DP-70 fluorescence microscope (Shinjuku-ku, Tokyo, Japan).

Uptake of IR-780 dye in prostate cancer cells

The cell staining procedures were described previously [5]. In brief, cell suspensions of PC-3, LNCaP, and RWPE-1 (1×10^4/well) were placed into 4-chamber slides (Nalgen Nunc) and continued to grow for 24 h. After the removal of culture medium, working solutions of IR-780 dye (20 µM) were added. The slides were incubated at 37°C for 20 min, then washed twice with phosphate-buffered saline (PBS). Cells were counterstained using 4',6-diamidino-2-phenyindole (DAPI) at 37°C for 10 min, followed by washing twice in PBS and 10-min fixation with 4% paraformaldehyde (Sigma-Aldrich). Then the slides were covered with glass coverslips using aqueous mounting medium (Sigma-Aldrich) and observed under a confocal laser microscope using 633 nm as the excitation wavelength and 780 nm as the emission wavelength [7].

Subcellular localization of these dyes in prostate cancer cells were detected according to a previously established protocol [5]. Briefly, commercially available Mito Tracker Green FM probes (Molecular Probes, Camarillo, CA, USA) were used to track cytoplasmic mitochondria. After DAPI staining, slides underwent staining using Mito Tracker 200 nM for 30 min at 37°C, and then were rinsed repeatedly. After repeated washing and mounting, the slides were observed under a confocal laser microscope. Emission/excitation lights for Mito Tracker were 490 nm/516 nm. Images captured from the same visual field were merged for co-localization analysis of IR-780 dye.

IR-780 dye for NIRF imaging of prostate cancer

All animal experiments were conducted in accord with Animal Care and Use Committee Guidelines of the Fourth Military Medical University. Six specific-pathogen-free athymic nude mice and 18 BALB/C mice (4–6 weeks old and weighing 20–25 g) were obtained from the Experimental Animal Center of the Fourth Military Medical University, housed in normal light-dark cycle with free access to food and water. Human prostate cancer PC-3 cells (1×10^6) were implanted subcutaneously into 3 athymic nude mice pursuant to the procedures previously reported [12], and 3 athymic nude mice without inoculation were used as normal controls. When tumor sizes reached about 5–10 mm in diameter, as assessed by macroscopic observation, palpation, or X-ray, IR-780 dye was injected i.p. at a dose of 0.334 mg/kg. The mice were anesthetized 24 h after NIRF dye injection by 2% isoflurane in 100% oxygen with a delivery rate of 1.5 L/min. The whole-body NIRF imaging of normal and tumor-loaded mice was taken using a IVIS Lumina II imaging station (Caliper Life Sciences, Hopkinton, MA, USA). Then, tumor and organs were retrieved for bio-distribution study of IR-780. The subcutaneous xenografts were further used for histological analysis. Frozen sections and paraffin-embedded tissue sections were prepared for confocal imaging and HE staining. Briefly, for immediate confocal microscopy, tissues were embedded in OCT medium (Sakura Finetek, Torrance, CA, USA). Frozen sections (10-µm thickness) were cut and then stained by DAPI. Finally, mounted sections were observed under a confocal microscope to detect the NIRF signals. For hematoxylin and eosin (HE) staining, tissues were fixed in 4% paraformaldehyde and embedded in paraffin [13]. Sections (5-µm thickness) from paraffin-embedded tissues after HE staining were examined and imaged under a DP-70 microscope (Japan) by an expert pathologist.

To evaluate the acute toxicity of IR-780 dye, daily i.p. injection of 100 µL IR-780 dye at a dose of 0.334 mg/kg and 3.334 mg/kg to 6 BALB/C mice in each group was performed for 1 month. Six mice in the control group were injected with 100 µl normal saline. These mice were observed and weighed daily. One month later, mice were sacrificed and all tissues were retrieved for histological evaluation by HE staining.

Data processing and statistics

Numerical data are described as the average ±SEM. SPSS software 16.0 (SPSS Company, Chicago, IL, USA) was used for statistical analysis. The statistical significance of data between the 2 groups was determined by *t* test. P less than 0.05 was recognized as statistically significant.

Results

Dose-dependent inhibition of cultured prostate cells by IR-780 dye

Human prostate cancer cells PC-3, LNCaP, and normal human prostate epithelial cells RWPE-1 were selected to evaluate the

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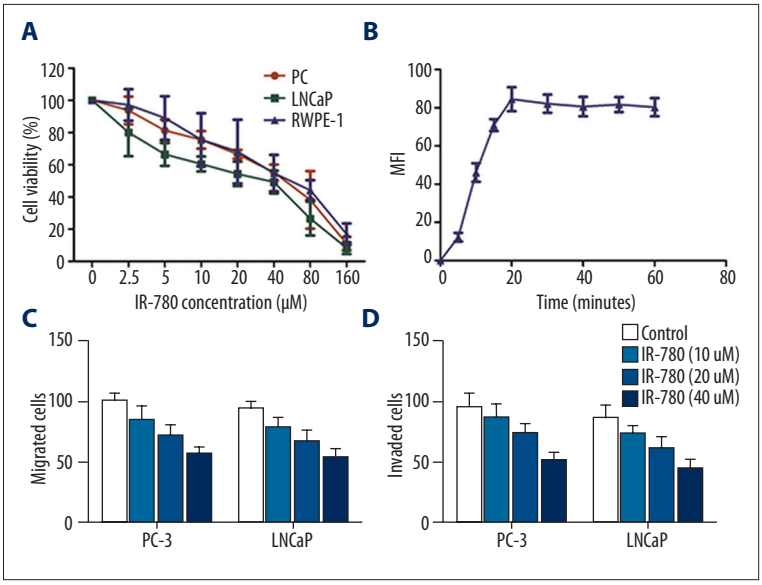

Figure 1. Optimization and characterization of IR-780 dye for prostate cancer cell imaging. (**A**) Cell viability test by MTT assay. Prostate cancer cells (PC-3 and LNCaP) and normal prostate epithelial cells (RWPE-1) were incubated with IR-780 dye at different concentrations (0, 2.5, 5, 10, 20, 40, 80, 160 μmol/L) for 24 hours. Dose-dependent inhibition of IR-780 dye on cell proliferation was revealed; (**B**) Mean fluorescence intensity of PC-3 cells stained with 20 μmol/L IR-780 dye at different time points; (**C**) Migration assay of PC-3 and LNCaP cells (1×10^4/well) after 24h IR-780 incubation (20 μmol/L). Migrated cells were stained with DAPI and counted; (**D**) Invasion assay PC-3 and LNCaP cells (1×10^4/well) with 24 h IR-780 incubation (20 μmol/L). Invaded cells were stained with DAPI and counted. Data were presented as mean ±SD of three independent experiments.* P<0.05.

impact of IR-780 dye on cell proliferation. We found that IR-780 at higher concentration strongly inhibited cell proliferation, irrespective of the malignant or normal phenotype of these cells. This effect was dose-dependent, as revealed by MTT assay (Figure 1A). IR-780 dye at 20 μM was selected for the optimization of staining time. Mean fluorescence intensity of PC-3 cells incubated with IR-780 rapidly increased, peaked at 20 min, and slowly reduced thereafter (Figure 1B). PC-3 and LNCaP cells treated with 20 μM IR-780 also displayed diminished capabilities in cell migration (Figure 1C) and invasion potential (Figure 1D), suggesting the potential role of IR-780 as a multifunctional dye with both imaging and tumor-killing ability.

Selective uptake and accumulation of IR-780 dye in prostate cancer cells

PC-3, LNCaP, and RWPE-1 were stained by 20 μM IR-780 dye for 20 min to evaluate whether the NIRF signal and uptake of IR-780 varies in prostate cancer cells and normal prostate epithelial cells. DAPI and NIRF images of the same visual field in each slide were taken under confocal microscopy (Figure 2). Selective uptake and accumulation of NIRF dyes was found in prostate cancer cells. There was only a weak NIRF signal in RWPE-1. These results further documented the unique property of IR-780 in cancer-targeted imaging.

Previous studies from other groups have demonstrated the preferential retention of IR-780 dye in the mitochondria of breast cancer cells and drug-resistant lung cancer cells [14]. We expanded the subcellular co-localization studies in prostate cancer cells PC-3 and LNCaP. Cells and mitochondria were clearly delineated by IR-780 and Mito-tracker dye staining (Figure 3A–3C). Merged images revealed a large overlap of cytoplasmic staining between IR-780 and mitochondria (Figure 3D), suggesting that a substantial portion of these dyes accumulate in the mitochondria of prostate cancer cells.

Inhibition of OATPs greatly affect the uptake of these dyes according to studies from varius research groups, but the explicit mechanism remains elusive [15,16]. Based on previous evidence of elevated OATP1B3 expression in prostate cancer, we employed OATP1B3 inhibitor to test whether this subfamily of OATPs exerts a central role in this context [8,17,18]. The selective OATP1B3 inhibitor, cholecystokinin octapeptide (20 μmol/L), was added to the chamber 5 min before the staining procedures. Selective OATP1B3 inhibitor cholecystokinin octapeptide significantly reduced the NIRF signal by IR-780, as revealed by decreased fluorescence intensity of NIRF signal (Figure 3E–3H). These results suggest that selective uptake of these dyes relies mainly on the transporting function of OATP1B3.

NIRF imaging of prostate cancer in mouse models using IR-780 dye

Subcutaneous models of prostate cancer using athymic nude mice were established to validate *in vivo* IR-780 dye application in prostate cancer detection. High signal-to-background ratios of xenografts were achieved in these mouse models 24 h after NIRF dyes administration, (Figure 4A). The bio-distribution study of IR-780 confirmed that its metabolism was mainly through the liver, since strong NIRF signals was detected in the gallbladder and feces, but only a weak signal was detected in the in bladder (Figure 4B). The NIRF signal in the tumor with 1 dose of IR-780 could still be detected at over 1 week later. Of note, strong near-infrared fluorescence was detected

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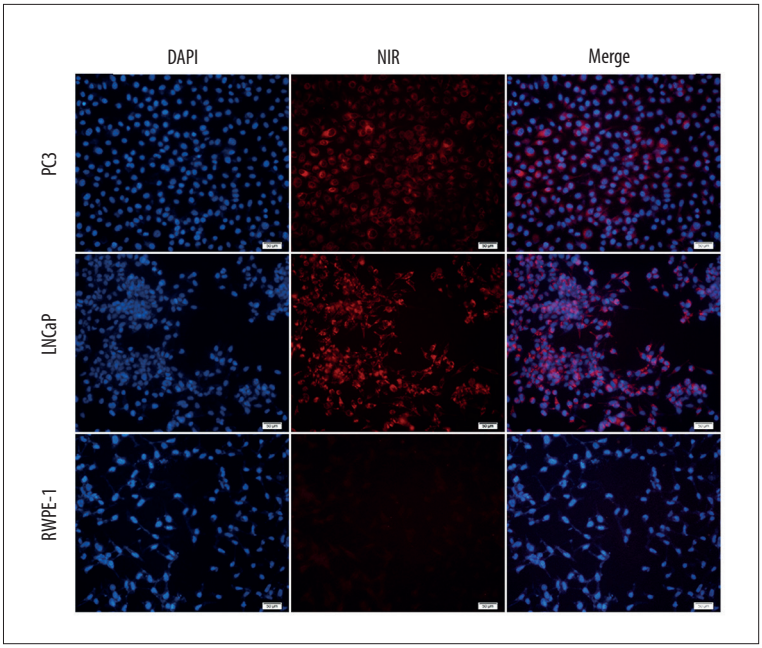

Figure 2. Preferential uptake of IR-780 dye in PC-3 and LNCaP cells. Intensive fluorescence was observed in prostate cancer cells,while mild fluorescence signal was detected in normal prostate epithelial cells RWPE-1 (magnification at ×200). DAPI was used for nuclei staining (blue).

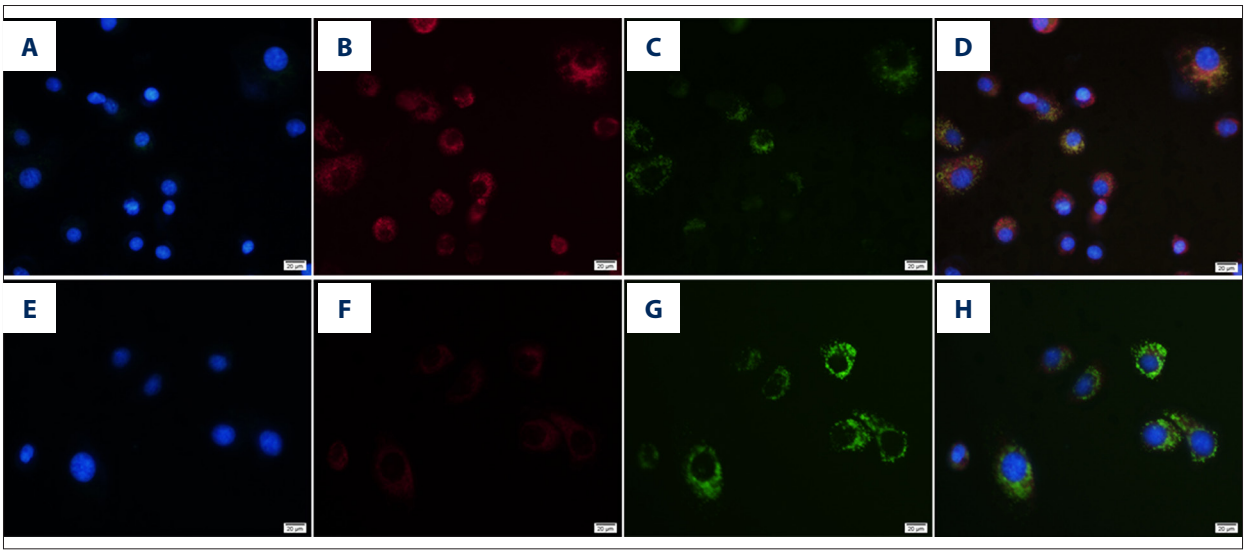

Figure 3. Subcellular co-localization study of IR-780 dye in PC-3 cells. (**A**) DAPI; (**B**) IR-780; (**C**) Mito Tracker; (**D**) Merged image of **A**, **B** and **C**; (**E**) DAPI; (**F**) CCK-8+IR-780; (**G**) Mito Tracker; (**H**) Merged image of **E**, **F** and **G** (magnification at ×400).

in frozen sections of xenografts retrieved immediately from sacrificed mice (Figure 4C–4E). HE staining of these tissues confirmed the existence of prostate cancer (Figure 4F). The long-lasting NIRF signal demonstrated the stability of IR-780 staining in prostate cancer, showing great promise for fast and accurate histological confirmation via frozen section during surgery. We tested a 10-fold higher dose in mice to observe the toxicity of IR-780. Daily i.p. injection of IR-780 dye to BALB/C mice at a dose of 3.34 mg/kg for 1 month resulted in no significant toxicity. Mice in both groups experienced no death or weight loss. Tissues at 1 month revealed no pathological change, as confirmed by HE staining.

Discussion

Various fluorescent nanoparticles have been constructed as potential contrast agents or vehicles for drug delivery to tumor sites, largely due to the convenience of NIRF imaging. However, conventional NIRF probes are vulnerable to photobleaching and self-aggregation, with limited signal-to-noise ratio and poor optical characteristics for clinical use. The pharmacokinetics of these NIRF probes are related with their concentration, surface coating, and chemical composition. For example, quantum dots are challenged by the potential toxicity of the metal core, relatively large size, and short circulation time [4].

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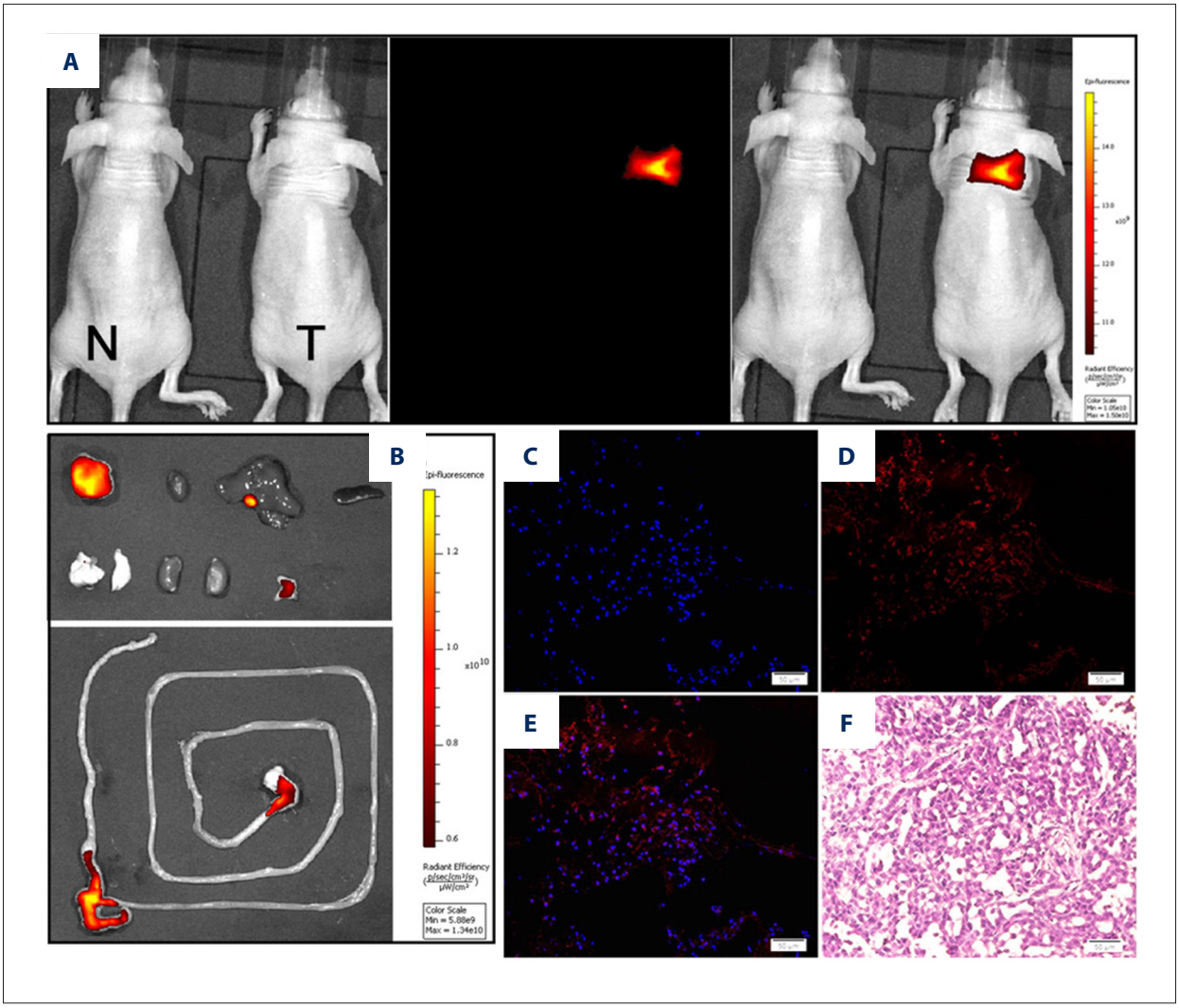

Figure 4. Near-infrared fluorescence (NIRF) imaging of prostate cancer xenografts using IR-780 in subcutaneous prostate cancer model. Frozen sections were prepared using freshly harvested xenografts from tumor bearing mice for immediate detection of NIRF signal. Remaining tissues were paraffin-embedded and prepared for HE analysis. (**A**) From left to right: bright light field, NIRF field and merged image (N: normal control, T: tumor bearing); (**B**) Bio-distribution of IR-780 in nude mice bearing prostate cancer (From upper left to bottom right: tumor, heart, liver, spleen, lung, lung, kidney, kidney, bladder, intestine); (**C**) DAPI staining of frozen sections; (**D**) NIRF signal were detected from frozen sections; (**E**) Merged image of **C** and **D**; (**F**) HE staining confirmed the existence of cancer tissue (magnification at ×200).

Organic dyes with emission in the near-infrared region have attracted increasing attention in recent years for diagnostic bio-imaging application. Deep penetration, up to 10 cm into tissues, makes NIRF imaging superior to traditional fluorescence illumination without safety concerns about radiation exposure [19,20]. However, the lack of specific targeting properties limits further biomedical application. Innovated strategies in the reconstruction or bio-conjugation of these dyes to produce targeted dyes are frequently utilized. A pool of targeting moieties, including antibodies, peptides, proteins, aptamers, and small receptor ligands, have been employed in multiple attempts [21,22]. Of note, IR-780 dye is associated with preferential accumulation in certain cancer cells, including breast cancer MCF-7 and lung cancer A549, superior to other common NIRF dyes as ICG for biomedical imaging [5]. Moreover, IR-780 dye displayed dual imaging and tumor-killing ability in prostate cancer cells, in agreement with previous findings by another research group [6].

The underlying mechanism of NIRF dye uptake remains unclear, although the contribution of OATPs in selective dye absorption was demonstrated [23]. The involvement of subtype proteins in the large OATP families is still unknown. Based on previous evidence of OATP1B3 up-regulation in prostate cancer cells, we investigated whether OATP1B3 exerts a dominant role in the NIRF imaging of prostate cancer cells but not

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normal prostate epithelial cells [24]. Thereafter, we revealed that the OATP1B3 subtype in OATPs family may be the transporter predominantly involved in the selective uptake of IR-780, consistent with the up-regulated OATP1B3 expression and function in prostate cancer [17,25].

In vivo imaging techniques allows for real-time observation of the target of interest. Fluorescence imaging via visible light in live animals is simple, cost-effective, and relatively sensitive [26]. However, observation in deep tissue is limited due to the poor visible light penetration. The fluorescence-based approach only allows for short-term labeling and imaging due to the high extinction coefficient of common fluorescent dyes. Strikingly, NIRF dyes enable tracking over longer periods without the loss of accuracy for diagnosis [7]. High efficiency in prostate cancer cell labeling could be realized using IR-780 dye, which might extend to the accurate quantification of live CTCs in prostate cancer patients [27]. Preferential accumulation of IR-780 dye in prostate cancer xenografts makes possible noninvasive and continuous monitoring of the tumor state as well as treatment outcome.

Conclusions

NIRF imaging using IR-780 dye can selectively identify prostate cancer cells as well as prostate cancer tissues in a tumor-bearing mouse model, which could extend to sensitive and reliable noninvasive cancer imaging. The limitation of NIRF imaging is the lack of detailed anatomical information, which could be solved by multifunctional NIRF probes that combine excellent NIRF dyes with other imaging modalities, such as MRI, CT, PET, and SPECT, as well as photoacoustic imaging (PAI) to provide anatomical information. This will be the next generation of NIRF dyes, an emerging field of cancer-targeted imaging technology and an integral part of biomedical research.

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