

NIR-II Imaging and Sandwiched Plasmonic Biosensor for Ultrasensitive Intraoperative Definition of Tumor-Invaded Lymph Nodes

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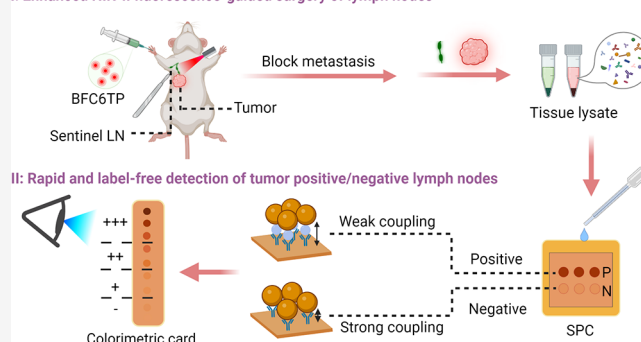
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ABSTRACT: Radical lymphadenectomy remains the cornerstone of preventing tumor metastasis through the lymphatic system. Current surgical resection of lymph nodes (LNs) based on fluorescence-guided surgery (FGS) suffers from low sensitivity/selectivity with only qualitative information, hampering accurate intraoperative decision-making. Herein, we develop a modularized theranostic system including NIR-II FGS and a sandwiched plasmonic chip (SPC). Intraoperative NIR-II FGS and detection of tumor-positive lymph nodes were performed on the gastric tumor to determine the feasibility of the modularized theranostic system in defining LN metastasis. Under the NIR-II imaging window, the orthotopic tumor and sentinel lymph nodes (SLNs) were successfully excised without ambient light interference in the operating room. Importantly, the SPC biosensor achieved 100% sensitivity and 100% specificity for tumor markers and realized rapid and high-throughput intraoperative SLN detection. We propose the synergetic design of combining the NIR-II FGS and suitable biosensor will substantially improve the efficiency of cancer diagnosis and therapy follow-up.

KEYWORDS: Modularized theranostic system, Sandwiched plasmonic chip, NIR-II fluorescence-guided surgery, Tumor-positive lymph nodes

I: Enhanced NIR-II fluorescence-guided surgery of lymph nodes



Surgical resection of tumor-positive lymph nodes (LNs) is an effective therapeutic strategy to prevent tumor metastases.^{1–3} Radical lymphadenectomy is becoming essential to increase the overall survival rate.^{4,5} However, the excess dissecting of normal LNs will lead to impaired lymphatic reflux and invasive follow-up procedures with the risk of complications.⁶ To improve the accuracy and safety of tumor-positive LN excision, it is critical for intraoperative detection of tumor-positive/negative LNs. In addition, because the lymphatic system generally goes along with blood vessels, it is important to avoid harming blood vessels and adjacent vital tissues during the surgery.^{7,8} Hence, accurate pre- or intraoperative diagnosis can assist surgeons in observing actionable information on the presence, progression, and treatment response of cancer.^{9–11}

The emergence of fluorescence-guided surgery (FGS) has substantially contributed to the field of cancer surgery by illuminating the tumor margins and/or tumor-adjacent LNs with a fluorescent fluorophore.^{12–14} FGS was further catalyzed by the near-infrared (NIR) imaging window (700–900 nm) given that NIR light provides improved penetration depth and spatiotemporal resolution.^{15–19} Surgeons will rely on the signals from NIR contrast agents to perform precise surgical

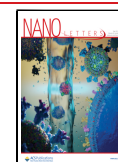
resection of the primary tumor and tumor-adjacent LNs. Imaging with an even longer wavelength in the NIR-II window (900–2000 nm) will usher FGS into a new paradigm shift, as the NIR-II transparent imaging window affords even higher imaging contrast at subcentimeter penetration depths.^{20–23} However, the use of the NIR-II contrast agent with bright/stable emission and fast excretion features is still rare, and it is necessary to enrich the library of clinically favorite NIR-II dyes to promote the clinical translation of NIR-II FGS.^{24–28}

In the field of tumor-targeted FGS, the antibody-based NIR probes (e.g., cetuximab-IRDye800^{29,30}) have shown improved sensitivity in identifying tumor regions.^{31–35} Despite the excitement, only qualitative imaging instead of quantitative is achieved, failing to provide accurate intraoperative decision-making in radical lymphadenectomy.^{36,37} Due to the sensitivity

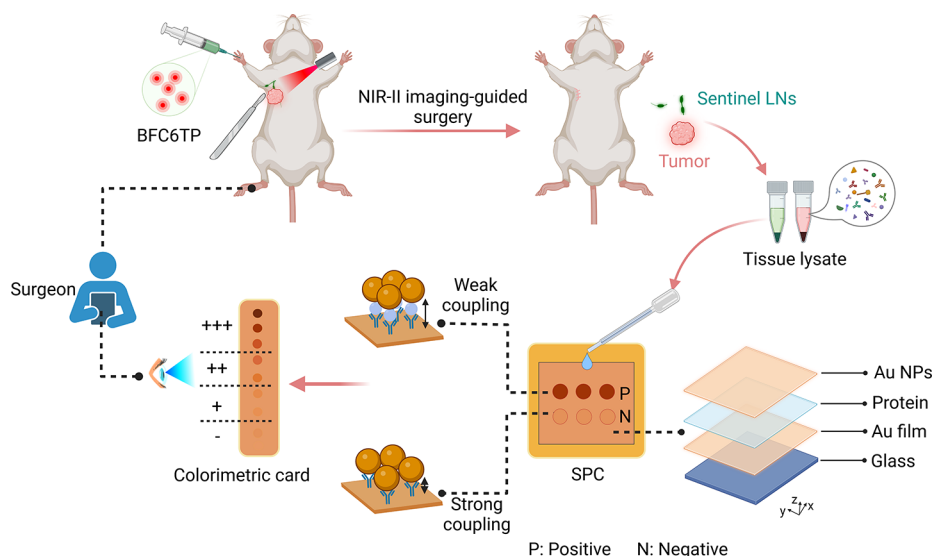
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Scheme 1. Schematic Illustration of the Intraoperative Theranostic System, Including NIR-II Imaging-Guided Surgery and Rapid Label-Free Detection of Tumor-Draining Lymph Nodes (LNs)⁴⁴



⁴⁴BFC6TP was designed to match the surgical navigation of the tumor and adjacent LNs in the NIR-II window. The obtained tumor-positive/negative LNs were detected using the SPC platform. Breaking through the complicated pathological examination, the results can be output by the naked eye and help surgeons to decide whether to resect higher-tier nodes and/or provide timely feedback for further treatment decision-making. The schemes were created using the online software: <https://biorender.com>.

limits, the signals of potentially slight metastases cannot be easily distinguished from the background signals.^{38–40} Although a lot of powerful and complementary technologies have contributed to the diagnosis of cancer, such as the biopsy, nanomedicine diagnosis system,⁴¹ reverse transcriptase-polymerase chain reaction (RT-qPCR),⁴² and immunofluorescence pathological analysis,⁴³ either sensitivity or specificity, convenience, timeliness, or production expense of current detection methods limits the effective intraoperative decision-making of whether to resect higher-tier nodes in radical lymphadenectomy. Therefore, it is extremely vital to develop a next-generation intraoperative immunoassay to rapidly determine the stages of resected sentinel LNs (SLNs). Several methods with ingenious optical labels and electric sensing devices have been massively studied, and several versatile optical plasmonic biosensors have also been used for virus or disease detection, which greatly improved the clinical performance of biomarker detection.^{44–49}

Hence, we develop a proof-of-concept intraoperative navigation system, enabling not only an enhanced NIR-II FGS of LNs but also a rapid detection of tumor-infiltrated LNs (Scheme 1). First, a bright and stable NIR-II dye (BFC6TP) with good biocompatibility and fast renal excretion is synthesized to achieve the NIR-II FGS of LNs. NIR-II imaging by BFC6TP can indiscriminately mark the tumor and SLNs with remarkable spatiotemporal resolution. Further, a sandwiched plasmonic chip (SPC)-based immunoassay with a visible diagnostic output signal for the rapid and label-free detection of tumor-positive LNs was applied. By changing the thickness of the immunobinding antibody layer (cetuximab and epidermal growth factor receptor (EGFR)⁵⁰ in this work) between Au nanoparticles (NPs) and Au films, the weakened electromagnetic coupling between the metal materials can be transduced into reflected visible light output signals, which could be visualized by the naked eye, or the captured antigens could be quantified using a microscope/smartphone. The SPC

platform with the thickness-sensing mechanism affords high-throughput antibody screening and rapid detection of micrometastasis tumor biomarkers. In this system, the NIR-II navigation system substantially improves outcomes of FGS-based tumor treatment by providing the precise dissection and identification (intraoperative decision-making) of tumor-positive LNs. Concurrently, the SPC platform also provides an unprecedented quantitative assessment of target expression for the FGS system, opening novel perspectives on the pre-, intra-, or postsurgery diagnosis.

Bright/stable signals and high biocompatibility are demanded in clinical navigation contrast agents. To this end, we developed an optimized probe with both photophysical stability and high quantum yield (QY) (Scheme S1) according to our previous protocol⁵¹ toward the shielding unit–donor–acceptor–donor–shielding unit (S-D-A-D-S) framework. Through rational donor engineering (hexyl-tert (ethylene glycol) furan), we accessed a bright NIR-II probe (BFC6TP) conjugated with two 1.5 kDa polyethylene glycol (PEG) chains (Figure 1a). The photophysical properties of BFC6TP exhibited an absorption peak at ~730 nm and NIR-II fluorescence emission (1050 nm emission peak with a tail extending into 1400 nm) (Figure S1). The NIR-II QY is up to 3%, almost 2-fold improvement compared to the previously reported S-D-A-D-S fluorophore (Table S1). Successful synthesis was also confirmed by the ¹H nuclear magnetic resonance (NMR) spectrum, ¹³C NMR spectrum, and high resolution electrospray ionization mass spectra (HRMS (ESI)) spectrum (Figure S2). The capillary penetration test indicated that BFC6TP exhibited a considerable penetration under the >1100 nm region compared with the clinically used cyanine contrast indocyanine green (ICG)^{16,52} and the inorganic NIR-II probe PbS QDs (Figure S3).

For *in vivo* use, biosafety is a crucial parameter for the clinical translation of imaging probes.⁵³ The cell viability test showed that above 80% of cells were maintained after 24 h of

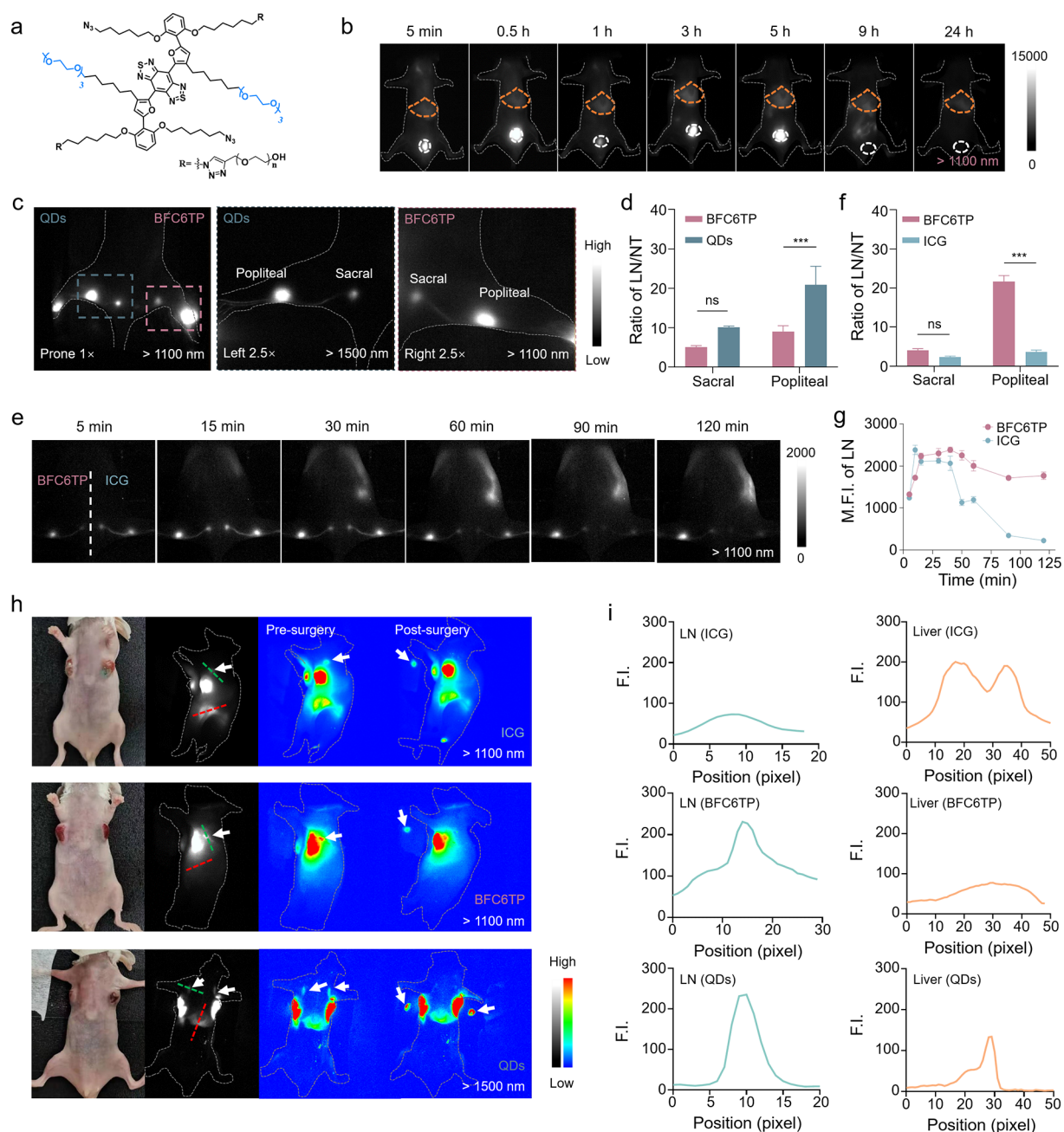


Figure 1. BFC6TP enabled high-quality NIR-II imaging of SLNs and imaging-guided resection of tumor SLNs. (a) Chemical structure of BFC6TP. (b) *In vivo* NIR-II imaging (1100 nm long-pass filter, 500 μ M (200 μ L), 50 ms exposure time, supine position) in selected time points after intravenously administering BFC6TP. (c) Whole-body (1 $\times$) and magnification location (2.5 $\times$) fluorescence images of popliteal and sacral lymph nodes after intradermal injection of QDs (2 μ M, 25 μ L, left) and BFC6TP (300 μ M, 25 μ L, right) in foot pads. (d) LN to normal tissue (LN/NT) ratios of BFC6TP and QDs groups. (e) Photostability images of BFC6TP and ICG-administered mice (500 μ M, 25 μ L). (f) LN/NT ratio of the 1.5 h time point of NIR-II LN imaging in (f). (g) Mean fluorescence intensity (M.F.I.) curves of LNs administered with BFC6TP and ICG. (h) NIR-II imaging and imaging-guided surgery of tumor-draining SLNs by using BFC6TP (500 μ M, 25 μ L), ICG (500 μ M, 25 μ L), and QD (2 μ M, 25 μ L) contrast agents. (i) Fluorescence intensity (F.I.) curves over the ROI in (h). Green dashed line, ROI of LNs; red dashed line, ROI of the liver.

incubation with BFC6TP of a high concentration (50 μ M) (Figure S4a). Compared with ICG, BFC6TP showed even higher stability in the hemolysis test (Figure S4b). For blood and tissue biosafety evaluation, the blood routine examination and liver/kidney indexes showed no significant change (Table S2 and Figure S5). The H&E staining also proved that no inflammation and tissue damage existed in all tested organs (Figure S6).

A favorable contrast agent displays the timely excretion from the body with minimum accumulation/retention in off-target tissues. The NIR-II imaging of BFC6TP showed an obvious fluorescence signal located in the bladder within 5 min. Then, the fluorescence signal showed depletion over time and nearly disappeared at the 24 h time point (Figure 1b). Taken together, the low organ accumulation of BFC6TP provides a benefit to the improved imaging contrast and fast renal

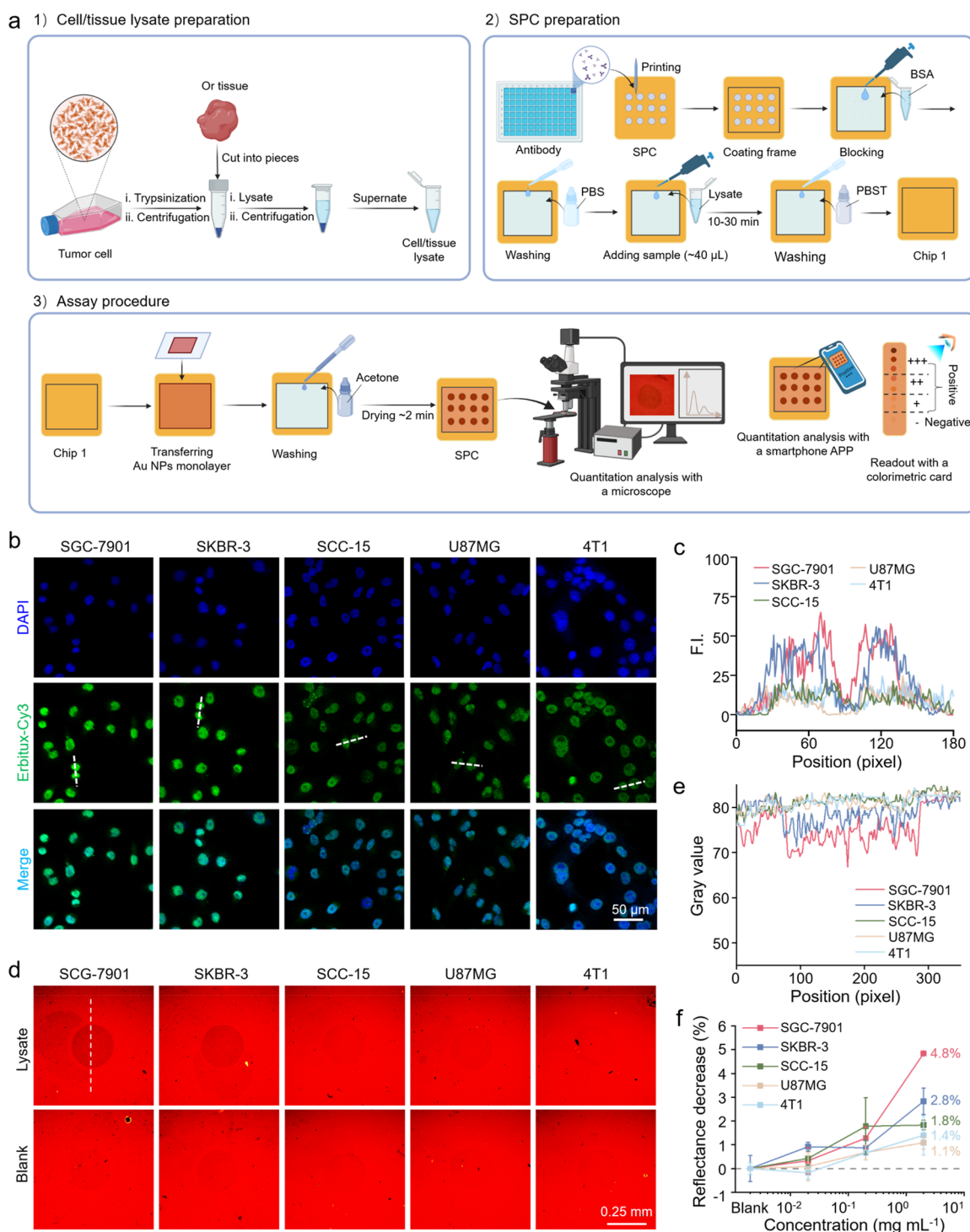


Figure 2. SPC immunoassay provided rapid label-free detection of EGFR-positive tumors. (a) Schematic depicting the SPC immunoassay fabrication steps and the detection procedure. (b) Screen of EGFR overexpressed cells by confocal images after being coincubated with Cy3-labeled Erbitux and (c) cross-sectional fluorescence intensity profile of five cell lines. (d) Microscope images of the resulting SPC after incubating with five cell lysates (2 mg mL^{-1}). BSA (Blank) was used as a negative control. The white dashed line indicates the measurement position of the spot. (e) Cross-section curves of resulting SPC after incubation of the cell lysate. (f) Peak intensity differences between cell lysates and the blank group.

excretion affords a benefit to the overall biosafety, primed for NIR-II FGS.

In vivo noninvasive fluorescence imaging of the lymphatic system has offered real-time modality to assess the lymphatic dysfunction or tumor-invaded LNs diagnosis. Stable photobleaching resistance is the key to high-quality intraoperative real-time imaging. As shown in Figure 1c,d, BFC6TP

performed bright LN imaging, and the higher LN-to-normal tissue (LN/NT) ratio of BFC6TP produced a competitive value to the NIR-IIb probe (QDs). Further, BFC6TP performed a stable LN imaging for 2 h in Figure 1e, and the popliteal LN/NT ratio of BFC6TP was nearly 5.4-fold higher than ICG with equivalent dosages after 1.5 h of continuous irradiation (Figure 1f). After 15 min of irradiation, The LN

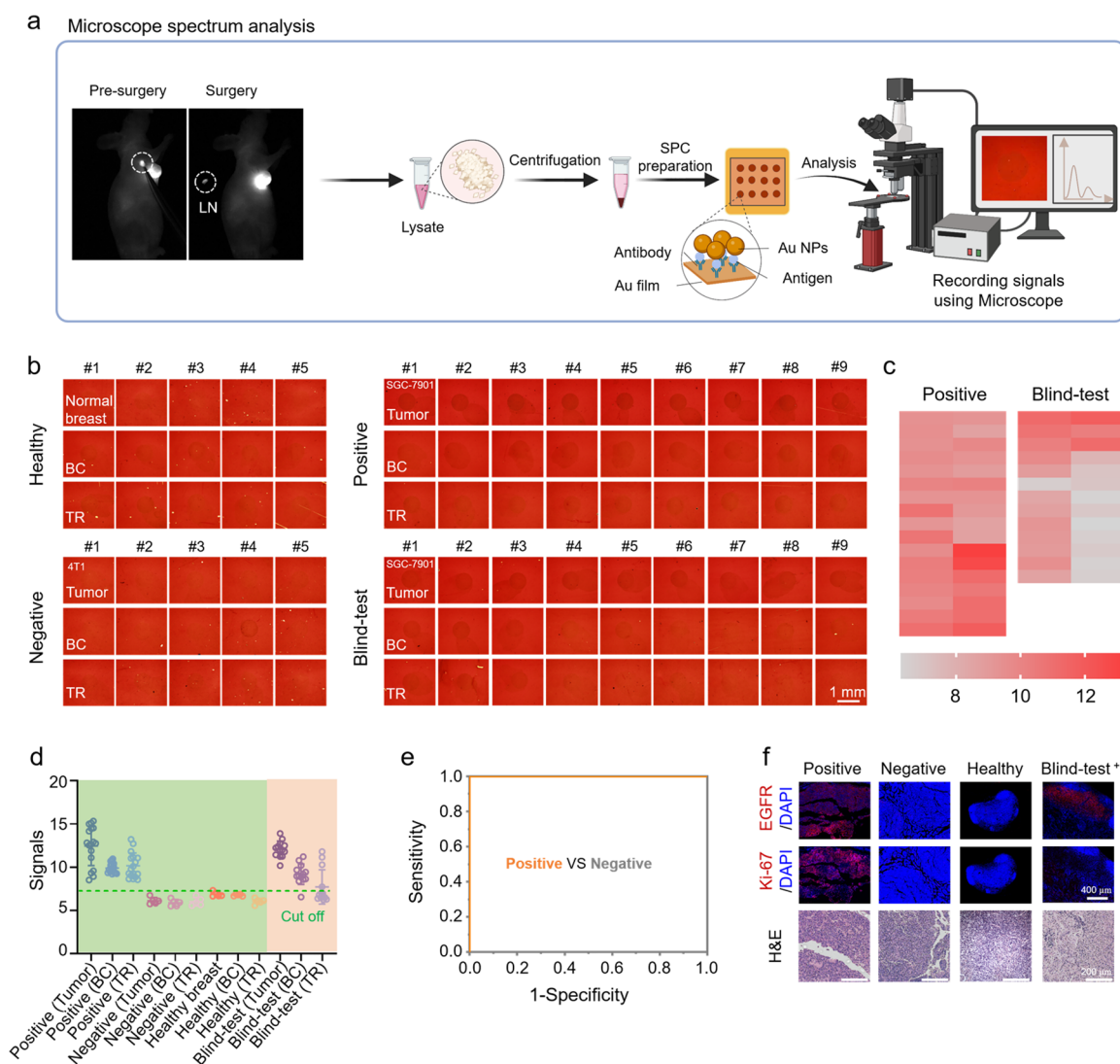


Figure 3. Intraoperative tumor-invaded SLNs detection. (a) Illustration of NIR-II imaging-guided surgery and label-free detection of tumor-infiltrating LNs. (b) Tumor and tumor-adjacent SLNs with different EGFR-expression levels were detected by the SPC platform. BC: brachial LN. TR: thoracic LN. (c) Heat map analysis of BC and TR LNs in the positive and blind-test groups. (d) Spectral analysis results collected using the optic fiber-equipped microscope. The cutoff line was determined by the average value plus double times SDs of the healthy LNs. (e) Receiver operator characteristic (ROC) curves generated using both EGFR-positive and -negative tumor samples with 100% sensitivity and 100% specificity. (f) The immunofluorescence and H&E staining of BC LNs from different groups.

intensity of the BFC6TP group remained constant, in contrast to the cohort of ICG administration, which exhibited severe photobleaching over time (Figure 1g).

Accurately and selectively removing SLNs can prevent cancer metastasis, simultaneously alleviating lymphoedema and postoperative complications. ICG has occupied most of the market of NIR imaging contrast agents for SLN surgery. To perform SLN imaging with BFC6TP, ICG, and QDs, these probes were intratumorally injected into the breast tumor, followed by NIR-II imaging of the lymphatic vasculature and nodes. After injection, probes migrated into the tumor-adjacent SLNs along with the lymphatic vasculature. Representative time points of 2–5 h postinjection showed apparent thoracic (TR) and brachial (BC) LNs (Figure S7 and Figure 1h). Plotting the signal across a linear cross-section over the liver and tumor-adjacent SLNs position resulted in the LN/NT ratios. Results suggested that only BFC6TP afforded an obviously lower liver signal compared with the cohorts of ICG and QDs (Figure 1i). Notably, in the duration of the

monitoring, the SLN signal of the BFC6TP and QD administered groups increased, and the LN/NT ratio of the BFC6TP group nearly matched the QDs group at the later time points (Figure S7c). In addition, BFC6TP also enabled the high-quality NIR-II imaging of distalis LNs with an intradermal injection near the tail base of mice, and both inguinal and brachial LNs were outlined with high spatiotemporal resolution (Figure S8). In general, BFC6TP is a promising NIR-II contrast agent.

Our NIR-II imaging navigation system enabled precise excision of the primary tumor and tumor-adjacent LNs, yet it is more critical to quickly inspect the nodes to determine the necessity of excising higher tier nodes. The pathological inspection is always demands preparing the slices and needs well-trained pathologists to produce an accurate report. As the concentration of tumor cells is relatively low in the early stage of tumor-invaded LNs, selectivity/sensitivity is essential to provide an accurate detection result. It is also difficult to distinguish tumor micrometastasis from other normal

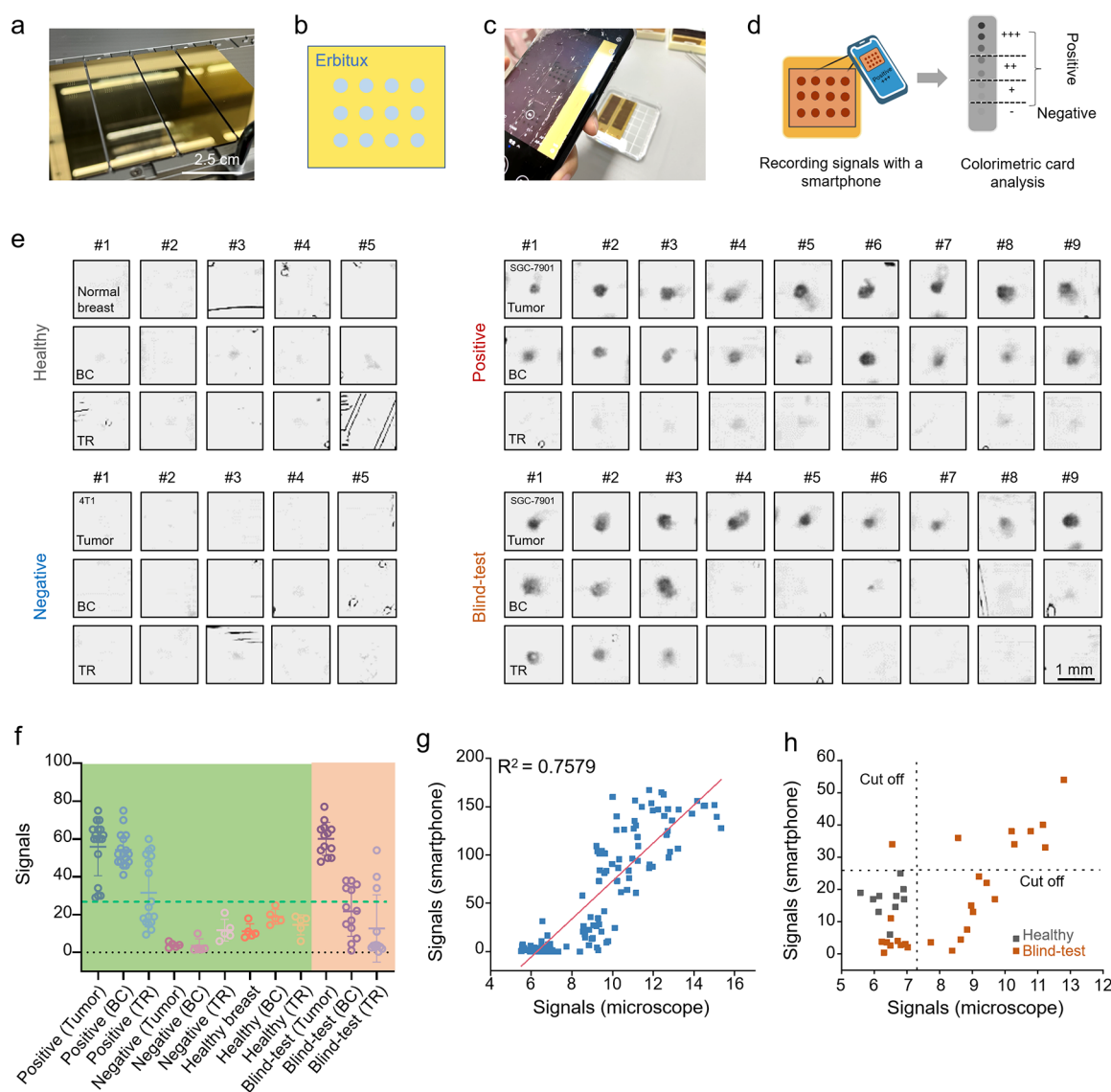


Figure 4. Naked-eye detection of EGFR-positive tumor and tumor-infiltrating LNs by SPC. (a) Digital picture of the Erbitux-printed Au films and (b) the printed layout. (c) Smartphone collection and (d) data analysis illustration by smartphone or naked eye (colorimetric card). (e) The gray value images collected by the smartphone using SPC-based immunoassays after detecting lysates from four cohorts in Figure 3b. Images in (e) were processed with a special algorithm for a preferable visualization. (f) The quantified signals from smartphone-collected images. (g) The correlation plot of signals from all samples between the microscope and smartphone readout. (h) The correlation plot of SLN signals from the blind-test group (brown) and healthy group (gray) recorded using the microscope and smartphone.

structures, and false-negative detection is a hidden danger in postoperative recurrence.⁵⁴ For this purpose, we developed an SPC immunoassay biosensor,⁵⁵ which provides fast/low-cost manufacturing steps and a visible diagnostic output signal, enabling the rapid and label-free detection of tumors with the microscope or naked-eye (smartphone) readouts in intra-operative tumor detection and tumor-invaded LNs detection. SPC could function as a powerful addition to the NIR-II imaging-navigation system.

The general structure of the SPC biosensor is comprised of the Erbitux (an antibody with high affinity to binding EGFR) printed gold film, a protein layer (cell or tissue lysate), and an Au nanoparticles (Au NPs) monolayer placed on the top (Scheme 1 and Figure 2a). Due to the distance-dependent electromagnetic coupling in sandwiched gold structures, a tiny thickness variation caused by immunobinding can be accurately detected and quantitatively transduced into visible

light output signals. We first selected five cell lines to detect the EGFR-expressed level (Figure 2b). Cy3-labeled Erbitux was used to quantify the EGFR level for these cell lines. The confocal images and profile curves exhibited that SGC-7901 and SKBR-3 had brighter Cy3 fluorescence signals, which were considered EGFR-positive cell lines (Figure 2c). Subsequently, lysates of five cell lines were collected and incubated on the Erbitux-printed Au film. After the necessary wash/dry steps and transfer of the Au NPs monolayer, the SPC assay was successfully performed (Figure 2a). Owing to the different EGFR-expression levels of the applied cells, the printed antibody would bind with different amounts of EGFR proteins, resulting in distinguishable reflected signals by microscope or naked-eye read-out. Microscopic images shown in Figure 2d,e revealed that SGC-7901 and SKBR-3 cells had positive signals (spots with a darker color). In addition, with increasing protein concentrations, the reflectance difference (between the spots

and the background) gradually increased and showed a blue shift (Figure 2f and Figure S9). These results proved that our SPC could provide quantification analysis for EGFR-positive tumor metastasis.

Guided by cell lysate data, we further selected an EGFR-positive cell line (SGC-7901) and an EGFR-negative cell line (4T1) to investigate the ability of intraoperative tumor detection and tumor-invaded LNs detection. We established 4T1 and SGC-7901 tumor models in breast pads of mice for 35–83 days thus allowing tumor cells to infiltrate into SLNs. Then, tumor and tumor-adjacent LNs were excised by NIR-II imaging-guided surgery, and the lysates of all excised tissues were tested using SPC immunoassays to quantify the EGFR level (Figure 3a). The normal breast pad and LNs were also collected as healthy groups ($n = 5$). Results indicated high positive signals of the SGC-7901 tumor (positive group, $n = 17$) and weak signals of the 4T1 tumor (negative group, $n = 5$) (Figure 3b). Notably, the SPC system can precisely report the EGFR-positive signals for tumor-adjacent LNs. A set of SGC-7901 tumor-bearing mice with uncertain tumor metastasis status were established for the blind-test group ($n = 13$), and both EGFR-positive and -negative LNs were observed. In contrast, both the 4T1 tumor group and the healthy group exhibited negative outcomes.

We next used an optic fiber-equipped microscope system to accurately quantify the optical output generated by the SPC assays. The quantitative signals from the spectral analysis assist accurately in reporting tumor-infiltrating LNs for both positive and blind-test groups (Figure 3c). EGFR-negative groups also showed obviously low signals compared to the positive group (Figure 3d). In addition, plotting the receiver operating characteristic (ROC) curve of SGC-7901 and 4T1 SPC assays resulted in 100% sensitivity and 100% specificity for EGFR-expressed tumors (Figure 3e). The pathological examinations of the excised LNs from four cohorts also confirmed the stages of tumor progression, which verified the diagnosis accuracy of our detection system (Figure 3f and Figure S10). Besides, the readout signal was still detectable after 18182-fold dilution of the original lysate protein, indicating SPC has superior sensitivity to tumor biomarkers (Figure S11a). For a shorter incubation time of 15 min, a stronger signal could be still detected, which can compare with the clinical rapid intraoperatively pathological inspection strategy (~20 min) (Figure S11b).

The SPC produced visible reflecting light signals are easily detected by various visible light readout devices. The fabrication procedure of the SPC platform is relatively convenient and cost-effective since only 5 nL of antibody is required for each SPC spot by a microarray printer (Figure 4a,b). During the detection of EGFR-positive tumors and tumor-draining SLNs, a smartphone was used to record pictures to check the ability of naked-eye observation and further analyze the signals through the established colorimetric card from standard samples (Figure 4c,d). The positive signals from the SPCs of four cohorts in Figure 3b were clearly distinguished by the naked eye (Figure 4e). The collected images by smartphone were further processed and quantified based on the corresponding gray value (Figure S12 and Figure 4f). The spectral data and the readout data by smartphone from all samples showed a reasonable correlation between the two methods (Figure 4g). The blind-test group also obtained similar positive/negative detection results by the two methods (Figure 4h). Collectively, the SPC platform serves as a

powerful diagnostic tool with supersensitivity (microscope, smartphone, or naked-eye readout) for intraoperative rapid decision-making of tumor/LN resection.

Most of the reported NIR-II fluorophores suffered from severe off-target organ retention and/or slow elimination rate, which seriously hampered the potential clinical use in humans.^{56,57} In this work, BFC6TP fluorophore showed almost no cellular toxicity *in vitro*, good *in vivo* biocompatibility, and rapid renal excretion ability. BFC6TP was proven to outperform the FDA-approved NIR fluorophore (ICG) to identify tumor-adjacent SLNs and imaging-guided SLNs dissection. Our BFC6TP also provided an accurate assessment of the lymphatic drainage tumor and distant metastases during tumor progression. The advances of the NIR-II navigation system coupled with the use of clinically favorite contrast agents will provide a better, safer, and more precise surgical procedure.

Despite of excitement of NIR-II imaging-guided surgery, it is still impossible to provide intraoperative decision-making given that NIR-II imaging cannot afford quantified information. We solved this issue by developing a rapid label-free SPC immunoassay, which adopted a novel thickness-sensing mechanism by sandwiching the protein interlayer, providing a supersensitive detection of the EGFR-positive tumor and tumor-infiltrating SLNs or higher-tier LNs. In comparison with pathological inspection, our platform has a similar short operation time but featured much-improved sensitivity and label-free readout. Due to the label-free high-throughput quantitative ability of our SPC, the next-generation SPC could integrate lots of well-known cancer targets in various cancer types.⁵⁸ But the sensitivity of smartphone or naked-eye output of our SPC is still not sufficient. Due to the difference in the types and molecular weights of sandwiched proteins detected in different cancers, nanoparticles with appropriate geometries, sizes, and densities are needed to greatly reflect the difference in thickness change and, thus, transduce effective thickness into a high reflectance in the SPC output signals. Hence, future studies will improve the sensitivity of the SPC by parameter optimization in terms of physical and chemical properties of Au nanoparticles.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.nanolett.3c00829>.

Experimental details for materials, characterizations, synthesis details of NIR-II fluorophores, and experimental methods; optical properties, NIR-II imaging ability, *in vivo* biosafety, and surgical navigation performance of BFC6TP; and details of the EGFR-positive cell screening, detection sensitivity, operation time, and processing methods of SPC (PDF)

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Author Contributions

#Y.W., J.N., and H.M. contributed equally to this work. S.Z., J.Z., and Y.W. conceived and designed the study. H.M. and Y.L. performed the synthesis and analysis of probes. Y.W. performed the main experiments. J.X. assisted with the animal imaging experiments. J.N. and Y.W. performed the intra-operative detection of tumor metastasis. Y.F.W. and F.G. provided the antibody and assisted in the cell imaging experiments. S.Z. and Y.W. wrote the paper, and all authors reviewed the paper.

Notes

The authors declare no competing financial interest.

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