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Sentinel lymph node mapping using near-infrared fluorescent methylene blue

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Methylene blue (MB) is a safe, low cost, and common blue dye remaining a popular choice for sentinel lymph node (SLN) mapping. However, the blue-dyed SLN in deep animal tissue could be seen only after a surgical management. Here the fluorescence properties of MB were investigated using a fluorescence spectrometer and a wavelength-resolved fluorescence spectral *in vivo* imaging system, and MB has been demonstrated for near-infrared (NIR) fluorescence mapping of SLN. When MB was injected intradermally into the second row breast of a rabbit, the lymphatic flow and axillary SLN could be observed directly through the NIR fluorescence emitted from the MB trapped in the deep tissue, which would eliminate the need for surgical management, and this fluorescence without any radioactivity was retained in the SLN for hours. The node also could be identified synchronously by the blue color and fluorescence of the MB after a surgical management. Using MB NIR fluorescence for SLN mapping may have great advantages over the traditional method.

[**Key words:** Methylene blue; Near-infrared fluorescence; Sentinel lymph node; *In vivo* imaging; Mapping]

3,7-bis(Dimethylamino)phenazathionium chloride, more often referred to as methylene blue (MB), has been extensively and safely used for over a century in the fields of diagnostics and disease therapy. Sentinel lymph node (SLN) mapping for cancers is one of the important applications of MB in clinical applications. During the past few years, MB has been successfully used in the localization of SLN for breast cancer (1–8), malignant melanomas (9, 10), thyroid cancer (11) and cervical cancer (12, 13), etc. Due to the increase in safety, equal efficacy, and decreased cost, MB has been demonstrated to offer an improved technique for SLN mapping over that of isosulfan blue (14) (another common blue dye remaining a popular choice for SLN mapping). Unfortunately, the SLN labeled with MB or other blue dyes in deep tissues can be observed only after a surgical management. Although SLN in deep tissues labeled with isotope can be directly detected without a surgical management, or can be detected usually by combining blue dye and isotope, the radioactive harm of the isotope should be cautiously considered. In addition, SLN biopsies are quite often very difficult to perform accurately in individual patients (15). Anything that potentially improves the technique is therefore welcome (15).

It is known that MB is a kind of near-infrared (NIR) fluorescent material. We found that although the fluorescent efficiency of MB was very low, its fluorescence could be captured by a high-resolution and high sensitivity scientific-grade charge-coupled device (CCD) camera equipped in an *in vivo* imaging system, and its fluorescent intensity was stable and bright enough for *in vivo* fluorescent imaging. We demonstrated here that the NIR fluorescence of MB could be used for SLN mapping, and the blue-dyed SLN of rabbit could be observed directly through deep tissue without a surgical management. This new technique has great advantages in SLN mapping over the traditional method.

MATERIALS AND METHODS

MB powder was obtained from Sinopharm Chemical Regent Co., Ltd. (in China). 100% fetal bovine serum (FBS) was obtained from Hangzhou Sijiqing Biological Engineering Materials Co., Ltd (in China). A fresh pig skin (~3.5 mm) was obtained from a food market. Female New Zealand rabbits (2.5–3 kg) were obtained from the Second Military Medical University and used in accordance with approved institutional protocols from the Shanghai Department of Experimental Animals Management. The rabbits were anesthetized with an ear vein injection of choral hydrate (10%) at a dose of 300 mg/kg, and the breast hair was then carefully shaved and further removed by a cosmetic grade hair removal cream. During the several-hour operation, the animals were maintained under anesthesia by re-injection of choral hydrate.

The UV–Vis absorption spectrum was taken on a diode array spectrophotometer (UV-2102PC, Unico (Shanghai), USA) with a deuterium lamp source. The excitation and emission spectra of MB were measured using a fluorescence spectrometer (F-2500, Hitachi, Japan) equipped with a xenon lamp source and a low-constant temperature bath (Shanghai Hengping Instrument Factory). To detect the fluorescence stability, the phosphate buffer solution (PBS) (pH 7.2)-dissolved MB and 100% serum-dissolved MB solutions were placed in 1 cm quartz cuvettes, respectively, and the open ends of the cuvettes were sealed with sealing film (Whatman). The cuvettes were placed in the sample pool of the fluorescence spectrometer. The samples in the cuvettes were retained at 37 °C and continuously excited at 665 nm for 90 min. During the irradiation process, the fluorescent spectra of the samples were measured.

The wavelength-resolved fluorescence spectral imaging was carried out using a spectra imaging system (Maestro™ *In Vivo* Imaging System, CRI, Inc., Woburn, MA, USA) comprising an optical head that includes a liquid crystal tunable filter (LCTF,

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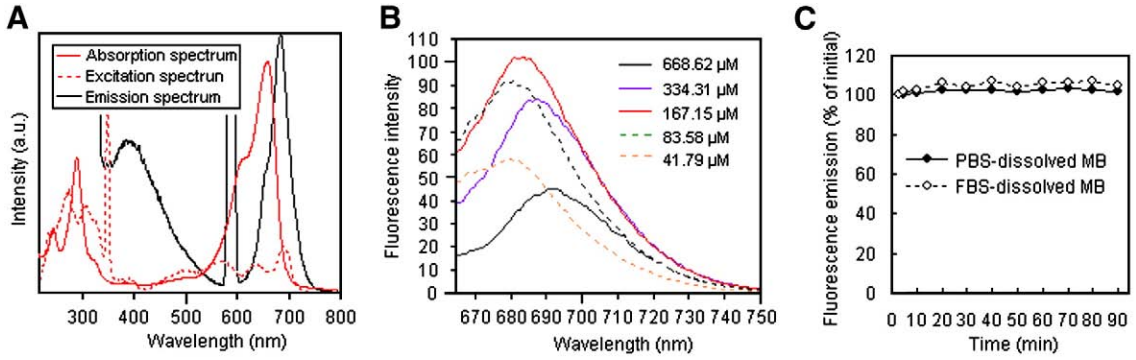

FIG. 1. Optical properties of the MB (Sinopharm Chemical Regent Co., Ltd) solutions. (A) Absorption (red solid curve), fluorescent emission (black solid curve, 292 nm excitation) and excitation (dashed curve, attributed to 700 nm emission) spectra of the PBS (pH 7.2)-dissolved MB solution (167.15 μM). (B) Fluorescent emission spectra (665 nm excitation) of the PBS solutions containing different concentrations of MB. (C) Fluorescence stability of 167.15 μM MB in PBS (solid curve) and in 100% FBS (dashed curve) with continuous excitation of 665 nm at 37 °C over time.

with a bandwidth of 20 nm and a scanning wavelength range of 500–950 nm) and a high-resolution and CCD imaging sensor. (1) For *in vitro* imaging of the PBS-dissolved MB solutions, the excitation band pass filters of 445–490 and 671–705 nm were used, respectively, and the corresponding emission band pass filters of 515 and 750 nm (long-pass) were used, respectively. The tunable emission filter was automatically stepped in 10-nm increments from 515 to 750 nm (for the excitation filter 445–449 nm, emission 515 nm (long-pass)) or 720 to 950 nm (for the excitation filter 671–705 nm, emission 750 nm (long-pass)) while the camera captured images at each wavelength with constant exposure. Overall acquisition exposure time was about 35 s. (2) For lymphatic mapping and SLN identification, 0.5, 0.8 and 1 ml of PBS-dissolved MB solution (5.34 mM (0.2%), 13.37 mM (0.5%), 26.74 mM (1%) and 53.48 mM (2%)) was injected intradermally into the second row breast of rabbits. The

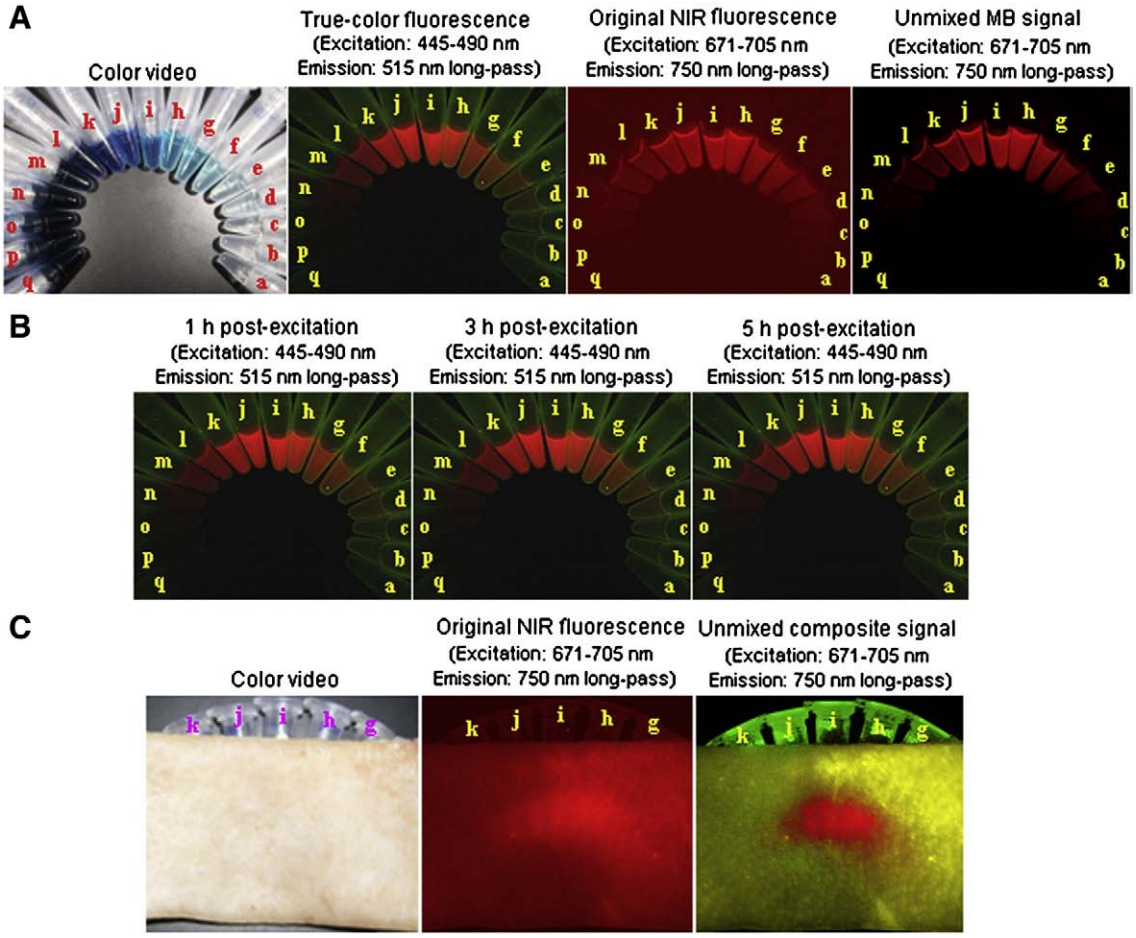

FIG. 2. *In vitro* images of the PBS-dissolved MB solutions. (A) Color video image (left) and NIR fluorescent images (the others) of the MB solution excited with different wavelengths. The NIR fluorescence images with 445–490 nm excitation and 515 nm emission (long pass) (second image) and with 671–705 nm excitation and 750 nm emission (long pass) (third image). And the unmixed MB NIR fluorescence image was analyzed according to the third image using the Maestro software. (B) Fluorescence stability of the PBS-dissolved MB solutions continuously irradiated at 445–490 nm for 1 h (left), 3 h (middle) and 5 h (right). (C) Images of the PBS-dissolved MB solutions shielded with a hairless pig skin (~3.5 mm). From left to right: color video (left), NIR fluorescence with 671–705 nm excitation and 750 nm emission (long pass) (middle) and unmixed composite signal according to the middle image (right). The PBS solutions contained the following concentrations of MB: (a) 0, (b) 1.08 μM, (c) 2.16 μM, (d) 5.22 μM, (e) 10.45 μM, (f) 20.89 μM, (g) 41.79 μM, (h) 83.58 μM, (i) 167.15 μM, (j) 334.31 μM, (k) 668.62 μM, (l) 1.33 mM, (m) 2.67 mM, (n) 5.34 mM, (o) 13.37 mM, (p) 26.74 mM and (q) 53.48 mM.