



Gdański Uniwersytet Medyczny

Wydział lekarski

Rozprawa doktorska

**Biopsja węzła wartowniczego w czerniakach skóry z zastosowaniem właściwości fluoresencyjnych błękitu metylenowego oraz zastosowanie tego związku w chirurgii sterowanej obrazem.**

**Sentinel node biopsy in skin melanoma using fluorescence properties of methylene blue and the use of this compound in image-guided surgery**

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GDAŃSK 2022

## Podziękowania

Serdeczne podziękowania mojemu Promotorowi, dr hab. n. med. Karolowi Połom , za opiekę merytoryczną, pomoc podczas prowadzenia badań i redagowania niniejszej pracy, a także za wsparcie, cierpliwość i wielką życzliwość

Panu dr n. med. Jarosławowi Skokowskiemu za nieocenioną pomoc podczas realizowania badań i redagowania niniejszej pracy

Najwspanialszej żonie Natalii oraz dzieciom Antoninie i Janowi – dzięki wam każdy nawet najtrudniejszy dzień jest wspaniały.

Kochanym rodzicom Grażynie i Tadeuszowi.

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## **WYKAZ SKRÓTÓW:**

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MB – Methylene Blue – Błękit Metylenowy

SLND – Sentinel lymph node dissection – Biopsja węzła wartowniczego

NIR – Near infrared – Bliskie światło podczerwone

NIRF – Near infrared fluorescence – Fluorescencja bliskiego światła podczerwonego

IGS - Image guided surgery – Chirurgia sterowana obrazem

Tc- 99m - Technetium 99m - Technet 99m

SBR - Signal to background ratio – Stosunek sygnału do tła

## **Słowa kluczowe:**

Błękit metylenowy, Chirurgia sterowana obrazem, Fluorescencja bliskiego światła podczerwonego, Czerniak skóry, Biopsja węzła wartowniczego,

## **Key Words:**

Methylene Blue, Image guided surgery, Near infrared fluorescence, Skin Melanoma, Sentinel lymph node dissection,

## **ABSTRACT**

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### **Introduction:**

Methylene blue (MB) also known as methylthioninium chloride was one of the first widely used fully synthetic drug. It was discovered in 1876 by the German chemist Heinrich Caro<sup>1</sup>. Since its discovery, it has been used in medicine in various clinical situations, especially in surgery<sup>2,3</sup>.

This marker and indicator, is used intraoperatively in various situations, for instance in the visualization of the presence of intestinal, entero-urinary or bronchopleural fistulas<sup>4-8</sup>. It is also used in other fields apart from surgery, such as, in the treatment of methaemoglobinemia-induced encephalopathy or in dermatology in herpes labialis, eczema herpeticum, oral candidiasis and cutaneous leishmaniasis<sup>2,9-14</sup>. This substance also has applications in treatment of cyanide poisoning. In the past, it was used in urinary tract infections as an antiseptic and stimulating the mucosa surface drug<sup>4,15</sup>. Nowadays, the main use of methylene blue is sentinel node biopsy (SNB) in various cancers<sup>16-20</sup>. SNB was first described in the beginning of nineties by Morton and Giuliano for melanoma and breast cancers. In recent two decades the development of image-guided surgery (IGS) has resulted in the use of more and more dyes that have fluorescent properties. It is natural to use known compounds with properties like MB.

MB, which is a thiazide dye, is very safe for patients. It can be used orally, intravenously or administered intradermally. Depending on the application, it is administered in various concentrations. Intravenous use should be slow and last between 3 - 10 minutes<sup>21,22</sup>. Occasionally, a temporary decrease in the pulse oximetry reading may occur when methylene blue is administered intravenously. This phenomenon is apparent and has no real relationship with the decrease in oxygen saturation of hemoglobin. This is due to a distorted pulse oximeter reading. After intravenous administration its half-life is 5-6.5 hours and it is excreted in the urine after about 4 to 24 hours<sup>15</sup>. Renal failure is no indication for decreasing the intravenous dosage, nevertheless caution should always be exercised<sup>14</sup>. Patients with hepatic insufficiency also do not require dosage changes<sup>21</sup>. After many years of using MB, the side effects are well known. Possible side effects of MB include: slight pain, burning, rash, abscess, necrosis and even ulceration at the site of intravenous injection<sup>4,23-25</sup>.

Sometimes the urine is green on the day it is administered<sup>4</sup>. Allergic reactions are concentration-related. They may be more common at doses above 5 mg / kg<sup>26-28</sup>. Therefore, the lowest effective dose should be used. A dose below 2 mg / kg is very safe for the patient<sup>29</sup>.

IGS is a new and growing element in modern surgery. Near-infrared (NIR) imaging is a technique that uses NIR light for intra-operative imaging of anatomical structures that are invisible under normal light. NIR fluorescence (NIRF) requires the use of a fluorophore and an imaging system to detect and measure its fluorescence upon excitation with an infrared light beam<sup>30</sup>. Fluorescent dyes can be applied locally or systemically as needed during surgery. This allows real-time evaluation. The fluorophore is excited by a specific wavelength of infrared light, and then using the imaging system the length of the reflected light, which is longer, is measured<sup>31</sup>. The range of the near-infrared light is 700-900 nm and enables to distinguish structures about 1 cm deep in the tissue<sup>32,33</sup>. NIRF imaging systems enable the selection of different colors with a fluorescent signal of a specific length, which helps in the intraoperative location of the structures. An essential element for the assessment of NIRF in most of the available equipment on the market is the need to dim the lighting in the operating room.

Methylene blue is not recognized as a perfect fluorophore due to the fact that it has a visible light emission spectrum in the range of 400-700 nm. MB excitation peak is about 700 nm - excitation 668 nm, emission 688 nm<sup>29,34</sup>. MB has a hydrophobic character which results in less tissue penetration with more background autofluorescence. The use of the fluorescent blue properties is sometimes impossible because some patients metabolize into the non-fluorescent form - leucomethylene blue<sup>15</sup>.

Skin melanoma is a malignant neoplasm. Occurrence frequency has been increasing in recent years<sup>35</sup>. The development of diagnostics, surgical treatment and, in particular, systemic treatment made it possible to achieve better treatment outcomes. It also reduces the scope of surgical procedures performed on patients over the last decades in the direction of less invasive. A perfect example of this procedure is the commonly performed sentinel lymph node dissection (SLND) in patients without clinical signs of lymph node metastases<sup>36</sup>. An updated indication for SLND includes melanomas in the stage greater than pT1a. SLND is a common and

basic element of surgical treatment of melanoma. Due to the possible localization of melanomas all over the skin, searching for the sentinel node may cause intraoperative problems. Nodal status assessment is an essential element of further treatment, since micrometastases may occur in up to 25% of clinically negative lymph nodes<sup>18,37</sup>. The first reports on performing a sentinel biopsy date back to 1992<sup>18</sup>. Morton and colleagues used blue dye to identify the node. In 1993, the first reports on the location of the sentinel node using a radiotracer appear<sup>38</sup>. In 1996, Albertini and collaborators described the combination of both methods for sentinel node identification. Currently, the gold standard in the performance of SLND is the dual method of determination - dye and isotope. The simultaneous use of both methods increases the probability of correct node detection to 96-99%<sup>39</sup>. With the development of new surgical techniques such as NIRF in 2005, Kitai and colleagues described sentinel node identification using fluorescent properties of indocyanine green in breast cancer<sup>40</sup>. Due to the standard use of MB in SLND in melanoma, an attempt to use its fluorescent properties seems natural. So far, there have been no reports on this in the literature. With the standard double sentinel node detection method, the attachment of the NIRF system enables the use of the fluorescent properties of MB. In the last few years intraoperative fluorescence imaging presenting important results being one of the key elements in IGS.

This doctoral thesis consists of two papers, one review paper about current trends and emerging future of methylene blue usage in a special attention in fluorescence prosperities, the second article is an original paper based on 20 melanoma patients who underwent SLND procedure using two standard dyes whit additional visualization of MB fluorescent properties.

### **Aims:**

#### Publication 1

The aim of this article is description of 5 major domains where the MB near infrared fluorescent guided imaging was used during surgical procedures.

#### Publication 2

The aim of the study was to present for the first time in clinical series of patient the usage of MB as not only a dye visualized with naked eye but also with fluorescent properties during sentinel biopsy procedures in melanoma patients.

## **Material and Methods:**

### Publication 1

A literature review of PubMed and Medline was conducted based on the historical, current and future usage of MB within the field of medicine. We reviewed not only the current usage but we also tried to grasp its function as a fluorophore in five main domains

### Publication 2

A prospective study of patients with melanoma of the trunk and extremities. The study was approved by the bioethics committee (no. NKBBN / 99/2018). The study included 20 patients who had standard indications for a sentinel node biopsy. In terms of sex, the groups were equal - 10 women and 10 men. All patients underwent the SLND procedure using a radioactive tracer and MB - standard naked eye visualization and fluorescence properties. The median age of the subjects was 60 years (range 21-85 years). In terms of melanoma localization, the study included 8 patients on the trunk, 3 on the upper limbs and 9 on the lower limbs. Melanoma advancement was in the range between pT1a to pT3b (patients with pTa1 were enrolled in the study if they had additional risk factors such as ulceration or mitoses  $\geq 1/\text{mm}^2$ ).

According to the SLND protocol at the Department of Oncology Surgery in Gdańsk, Medical University, each patient before the procedure (1-3 hours before skin incision) was given intradermally 1ml (1mCi/ml) technetium 99m (Tc-99m). MB 1ml (Metiblo 10mg /ml) was injected intradermally separately after induction of anesthesia and prior to prepping the patient.

During the procedure, the near infrared light imaging system NIR Quest Artemis (Quest Artemis, Netherlands) was used. This device includes a camera and an integrated NIR light source intended to aid in the imaging of MB as a fluorophore – excited wavelength of 680 nm, visualization at approximately 710 nm. The camera of the device was positioned approximately 20-30 cm from the patient's skin. Lymph node basin was examined with a handheld gamma camera system, Gamma Finder II (W.O.M World of Medicine GMBH; Berlin, Germany), prior to making a skin incision, along with assessment of the visibility of MB through the skin. The operating field was monitored by the NIR Quest Artemis camera throughout the duration of the

procedure. During the procedure, the surgeon had the opportunity to observe the camera image and operate in real time. It was necessary to dim the lighting in the operating room. Further, the nodes were inspected for radiation markers. Each node that was visible in one of the methods was treated as sentinel. The surrounding tissue was chosen as the background. A signal to background ratio (SBR)  $\geq 1.1$  with NIRF was considered positive. During imaging, a color image of the surgical field was visualized simultaneously as the NIRF to assist in surgical guidance.

The results of the study were presented in a statistic analysis such as the median with minimum and maximum values and the frequency expressed as a percentage. Chi-squared tests were used to test for differences between observed frequencies and frequencies that were expected under the null hypothesis. A P- value  $< 0.05$  was considered statistically significant. All statistical analyses were performed using the SPSS version 26.0 software package for Mac (IBM Corp, Chicago, IL, USA).

## **Results:**

### Publication 1

Utilizing the fluorescent properties of methylene blue, they can be used in five main topics. Visualization of ureters, parathyroid gland identification, pancreatic tumors imaging, detection of breast cancer tumor margins, as well as breast cancer sentinel node biopsy.

Intraoperative visualization of the ureter is possible using MB and NIRF system especial in laparoscopic procedure. Damage to the ureter during the procedure is a relatively rare complication, but it is associated with serious complications. Methylene blue excreted in urine after intravenous administration makes it possible to visualize the ureter in NIRF. Intravenous doses of MB are in a range from 0.25 mg/kg to 1 mg/kg.<sup>34,41</sup> The ureteral fluorescence was visible 10 minutes after the administration, even 60 minutes. In all of these dosages a significant difference has been shown between the mean signal-to-background ratio across all time points. Barennes et al. reported the highest mean SBR using the 0.75 mg/kg dose (mean = 5.29, SD = 2.72, 95% CI 4.84–5.75), and the lowest at 1 mg/kg (mean = 3.66, SD = 1.89, 95% CI 3.37–3.39)<sup>34</sup>. A limitation in the use of MB in the visualization of the ureter may be renal dysfunction

and the conversion of MB to non-fluorescent form, which is caused by the reduction and / or acidity of the environment.

MB has long been used in the intraoperative identification of parathyroid glands using doses of 3.5 to 7 mg / kg and changes in the color of the parathyroid glands seen with the naked eye<sup>42,43</sup>. Such high doses may be associated with the occurrence of side effects. NIRF makes it possible to visualize the parathyroid glands at lower doses. The lowest dose of methylene blue that has been administered intravenously to differentiate parathyroid glands from the surrounding tissue is 0.05 mg/kg, but the fluorescence only lasts for a few seconds, which makes it less than ideal for resections of the parathyroid glands<sup>19</sup>. Additionally, the parathyroid glands show autofluorescence at a wavelength of 750 nm to 785 nm<sup>44-46</sup>. The reason for this is unknown, it may be related to the histological structure and blood supply intraoperatively<sup>45,47</sup>.

The intravenous supply of MB also causes its accumulation in the cells of neuroendocrine tumors<sup>48,49</sup>. Due to NIRF, it is possible to visualize insulinomas with a diameter of less than 1 mm<sup>48,50</sup>. MB fluorescence can be used in the intraoperative identification of pancreatic PNET. The use of MB fluorescence in this case is under further research.

Tummers et al. used the fluorescent properties of MB to evaluate the margins of excised breast cancers during conserving surgery<sup>24</sup>. Preoperative intravenous administration of methylene blue at a dose of 1 mg / kg allowed to visualize the margins of the excised tumors using fluorescence NIR.

Jiang et al. demonstrated the possibility of using the properties of MB fluorescence in the identification of sentinel node in breast cancer<sup>23</sup>.

## Publication 2

Radioactive technetium was present in all cases 20/20 (100%). In 3/20 (15%) of patients, the sentinel node was visible through the skin by NIRF. The median SBR for the sentinel node visualization by fluorescence was 3,15 (range, 2,7 to 3,5). In 4 patients (20%), the fluorescence lymphatic channels were seen in NIRF before it was visible to the naked eye. The median SBR for lymphatic channel visualization by fluorescence was 3,69 (range 2,7 to 4,2). Sentinel nodes were visible by fluorescence with MB in 14

cases (65%). The median SBR for sentinel node visualization during lymphatic tissue preparation by fluorescence was 2.49 (range, 1.5 to 5.7). Only two patients who underwent SLND had metastases to these nodes, which required a total lymphadenectomy at the next operation. In both of these patients, the sentinel node was fluorescent. In 16 patients (80%) the lymph node was blue stained which allowed identification with the naked eye. In three of these patients, the sentinel node was blue, but no fluorescence was identified. In one patient, the lymph node showed fluorescence but no blue discoloration was visible to the naked eye. Of the four patients with fluorescence visible lymphatic channels, only one sentinel node was stained blue, but no fluorescence was detected. The relationship between the sentinel node fluorescence and factors such as gender, melanoma location and ulceration was also analyzed. No significant association was found between fluorescence and these factors. There were no adverse reactions associated with the use of methylene blue during the clinical study. There were also no serious surgical complications.

### **Conclusions:**

Publication1

Methylene blue is used in medicine for decades especially because of its accessibility, wide safety profile and procedure versatility. Using its fluorescent properties it showed wide variable application in different surgical techniques.

Publication 2

For the first time in a clinical setting the usefulness of fluorescent properties of methylene blue in sentinel biopsy in melanoma patients was described. Future studies are awaited to proof its clinical potential.

## **STRESZCZENIE**

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### **Wstęp:**

Błękit metylenowy (MB) znany również jako chlorek metylotioniniowy jest jednym z pierwszych szeroko stosowanych w pełni syntetycznych leków. Został odkryty w 1876 roku przez niemieckiego chemika Heinricha Caro<sup>1</sup>. W medycynie wykorzystywany jest często w różnych sytuacjach klinicznych w szczególności w chirurgii<sup>2,3</sup>. Znajduje zastosowanie w śródoperacyjnej wizualizacji\wykrywaniu obecności przetok jelitowych, jelitowo-moczowych czy oskrzelowo-opłucnowych<sup>4-8</sup>. Jest również wykorzystywany w leczeniu encefalopatii wywołanej methemoglobinemią. W dermatologii, w celu wspomagania gojenia opryszczki wargowej, wyprysku opryszczkowego, kandydozy jamy ustnej i leiszmaniazy skórnej<sup>2,9-14</sup>. Jest również używany w przypadku zatrucia cyjankiem. W przeszłości był stosowany w infekcjach dróg moczowych jako środek antyseptyczny i stymulujący działanie na powierzchni błony śluzowej<sup>4,15</sup>. Obecnie głównie jest wykorzystywany przy procedurze biopsji węzła wartowniczego -sentinel lymph node dissection (SLND) w wielu nowotworach<sup>16-20</sup>. SNB została po raz pierwszy opisana w latach dziewięćdziesiątych przez Mortoną i Giulano w przypadku czerniaka skóry i raka piersi. Rozwój chirurgii sterowanej obrazem w ostatnich latach doprowadził do zastosowania związków o właściwościach fluoresencyjnych do tej procedury chirurgicznej. Naturalne jest wykorzystanie znanych barwników przy SNB w poszerzeniu o ich właściwości fluoresencyjne.

MB to barwnik tiazydowy o bardzo bezpiecznym profilu. Może być podawany doustnie, dożylnie lub śródskórnie. W zależności od zastosowania jego stężenia są różne. Podawanie dożylnie powinno być powolne i trwać od 3 do 10 minut<sup>21,22</sup>. Czasami, po dożylnym podaniu błękitu metylenowego, może wystąpić przejściowy spadek odczytu pulsoksymetrii. Jest to zjawisko pozorne, które nie ma realnego związku ze spadkiem nasycenia hemoglobiny tlenu. Spowodowane jest to zaburzeniem odczytu pulsoksymetru. Po podaniu dożylnym jego okres półtrwania wynosi 5-6,5 godziny i jest wydalany z moczem po około 4-24 godzinach<sup>15</sup>. Nie ma potrzeby zmniejszania dawki dożylniej w niewydolności nerek, ale zawsze należy zachować ostrożność<sup>14</sup>. Pacjenci z niewydolnością wątroby nie wymagają zmiany dawkowania<sup>21</sup>. Po wielu latach stosowania MB skutki uboczne są powszechnie znane

i należą do nich: niewielki ból, pieczenie, wysypka, ropień, martwica, a nawet owrzodzenie w miejscu wstrzyknięcia<sup>4,23–25</sup>. Przy podaży dożylnej mocz może zmienić kolor na zielony. Reakcje alergiczne są zależne od stężenia, mogą występować częściej przy dawkach powyżej 5 mg/kg<sup>26–28</sup>. Dlatego należy stosować najniższą skuteczną dawkę. Dawka poniżej 2 mg/kg jest bardzo bezpieczna dla pacjenta<sup>29</sup>.

Chirurgia sterowana obrazem (IGS) to nowy i rozwijający się element nowoczesnej chirurgii. Obrazowanie w bliskiej podczerwieni (NIR) to technika wykorzystująca wiązkę światła podczerwonego do śródoperacyjnego obrazowania struktur anatomicznych, które są niewidoczne w normalnym świetle. Fluorescencja z zakresu promieniowania podczerwonego - near infrared fluorescence (NIRF) wymaga użycia ziązku z właściwościami fluoresencyjnymi oraz specjalistycznego systemu obrazowania do wykrywania i pomiaru jego fluorescencji po wzbudzeniu wiązką światła podczerwonego<sup>30</sup>. Barwniki fluoresencyjne można stosować miejscowo lub ogólnoustrojowo w zależności od potrzeb podczas zabiegu pozwalające na ocenę w czasie rzeczywistym. Związek fluoresencyjny jest wzbudzany przez określoną długość fali światła podczerwonego, a następnie za pomocą systemu obrazowania mierzy się długość wiązki światła odbitego, która jest dłuższa. Zasięg bliskiej podczerwieni wynosi 700-900 nm i pozwala na rozróżnienie struktur anatomicznych w badanej tkance na głębokości do około 1 cm<sup>32,33</sup>. Systemy obrazowania NIRF umożliwiają wybór różnych kolorów dla poszczególnych długości światła odbitego co pomaga w śródoperacyjnej lokalizacji struktur.

Błękit metylenowy nie jest uznawany za doskonały związek fluoresencyjny ze względu na to, że ma widmo emisji światła widzialnego w zakresie 400-700 nm. Pik wzbudzenia MB to około 700 nm – (wzbudzenie 668 nm, emisja 688 nm)<sup>29,34</sup>. MB ma charakter hydrofobowy co powoduje mniejszą penetrację do tkanek przy większej auto fluorescencji tła. Wykorzystanie właściwości fluoresencyjnych błękitu jest czasem niemożliwe, ponieważ niektórzy pacjenci metabolizują go do postaci niefluorescyjnej – błękitu leukoetylenowego<sup>15</sup>.

Czerniak skóry jest nowotworem złośliwym, którego częstość występowania wzrasta w ostatnich latach<sup>35</sup>. Rozwój diagnostyki, leczenia operacyjnego, a w szczególności leczenia systemowego umożliwia uzyskanie lepszych wyników leczenia. Zmniejszeniu ulega także zakres zabiegów chirurgicznych wykonywanych na przestrzeni ostatnich dziesięcioleci w kierunku mniej inwazyjnych. Doskonałym

przykładem tej tendencji jest powszechnie wykonywana biopsja węzła wartowniczego (SLND) u pacjentów bez klinicznych objawów przerzutów do węzłów chłonnych, umożliwiająca w dużej grupie chorych uniknięcia całkowej limfadenektomii<sup>36</sup>. Aktualne wskazanie do SLND obejmuje czerniaki w stadium większym niż pT1a (klasyfikacji TNM czerniaka). SLND jest powszechnym i podstawowym elementem chirurgicznego określenia stanu zaawansowania czerniaka. Ze względu na możliwą lokalizację czerniaków na całej skórze ciała poszukiwanie węzła wartowniczego może powodować problemy śródoperacyjne. Ocena stanu węzłów jest niezbędnym elementem dalszego leczenia, mikro przerzuty mogą wystąpić nawet w 25% klinicznie niepodejrzanych węzłach chłonnych<sup>18,37</sup>. Pierwsze doniesienia o wykonaniu biopsji węzła wartowniczego pochodzą z 1992 roku. Morton i współpracownicy użyli niebieskiego barwnika do identyfikacji węzła. W 1993 roku pojawiły się pierwsze doniesienia o wykorzystaniu znacznika połączonego z radioaktywnym izotopem<sup>38</sup>. W 1996 roku Albertini i współpracownicy opisali kombinację obu tych metod identyfikacji węzła wartowniczego która jest aktualnie złotym standardem w wykonywaniu SNB. Jednoczesne użycie tych metod zwiększa prawdopodobieństwo prawidłowego wykrycia węzła do 96-99%<sup>39</sup>. Wraz z rozwojem nowych technik chirurgicznych, takich jak NIRF w 2005 roku, Kitai i współpracownicy opisali identyfikację węzła wartowniczego przy użyciu zieleni indocyjaninowej w raku piersi<sup>40</sup>. Ze względu na standardowe zastosowanie MB w SLND w czerniakach, naturalna wydaje się próba wykorzystania jego właściwości fluoresencyjnych. Jednoczesne zastosowanie systemu NIRF podczas standardowej metody lokalizacja węzła umożliwia dołączenie do nich trzeciej związanej z fluorescencją błękitu. Jak dotąd w literaturze nie ma na ten temat doniesień.

Niniejsza praca doktorska składa się z dwóch artykułów. Jednej pracy przeglądowej dotyczącej aktualnych trendów i pojawiających się przyszłych zastosowań błękitu metylenowego ze szczególnym uwzględnieniem jego właściwości fluoresencyjnych. Druga to praca oryginalna oparta na 20 pacjentach z czerniakiem skóry, którzy przeszli standardową procedurę SLND poszerzoną o śródoperacyjną wizualizację węzła chłonnego wiązką światła podczerwonego w oparciu o właściwości fluoresencyjne błękitu metylenowego.

**Cele:**

## Publikacja 1

Celem artykułu jest przegląd dostępnej literatury oraz zebranie materiału na temat pięciu głównych zastosowań fluorescencji błękitu metylenowego podczas zabiegów chirurgicznych z wykorzystaniem obrazowania wiązką światła bliskiej podczerwieni.

## Publikacja 2

Celem pracy było wykonanie badania klinicznego u chory z czerniakiem skóry podanych biopsji węzła wartowniczego przy wykorzystaniu standardowej metody – radioizotop oraz błękit metylenowy poszerzonej o śródoperacyjną wizualizację węzła za pomocą systemu obrazowania wiązką światła bliskiej podczerwieni wykorzystując właściwości błękitu metylenowego.

**Materiały i metody:**

## Publikacja 1

Przeprowadzono przegląd literatury PubMed i Medline na podstawie historycznego, obecnego i przyszłego wykorzystania MB w medycynie. W szczególności skoncentrowano się na możliwych zastosowaniach właściwości fluoresencyjnych błękitu metylenowego w chirurgii sterowanej obrazem.

## Publikacja 2

Prezentuje prospektywne badanie dotyczące pacjentów z czerniakiem tułowia i kończyn. Badanie zostało zatwierdzone przez komisję bioetyczną (nr NKBBN/99/2018). Włączonych zostało 20 pacjentów leczonych w Klinice Chirurgii Onkologicznej Gdańskiego Uniwersytetu Medycznego ze standardowymi wskazaniami do biopsji węzła wartowniczego. Pod względem płci grupy były równe – 10 kobiet i 10 mężczyzn. Wszyscy pacjenci zostali poddani zabiegowi SLND z użyciem znacznika promieniotwórczego i MB - standardowa wizualizacja gołym okiem i ocena właściwości fluoresencyjnych. Mediana wieku badanych wynosiła 60 lat (zakres 21-85 lat). Pod względem lokalizacji czerniaka badaniem objęto 8 pacjentów ze zmianą

na tułowiu, 3 na kończynie górnej i 9 na kończynie dolnej. Stopień zaawansowania czerniaka waha się od pT1a do pT3b (do badania włączano pacjentów w stadium pTa1, jeśli spełniali dodatkowe czynniki w ryzyku, takich jak owrzodzenie lub mitoza  $\geq 1/\text{mm}^2$ ).

Zgodnie z protokołem SLND obowiązującym w Klinice Chirurgii Onkologicznej Gdańskiego Uniwersytetu Medycznego każdemu pacjentowi przed zabiegiem (1-3 godziny przed nacięciem skóry) podano śródskórnie 1ml (1mCi/ml) technetu 99m (Tc-99m). Dodatkowo wstrzyknięto śródskórnie MB 1ml (Metiblo 10mg/ml) po indukcji znieczulenia i przed rozpoczęciem operacji.

Podczas zabiegu zastosowano system obrazowania w bliskiej podczerwieni NIR Quest Artemis (Quest Artemis, Holandia). Urządzenie to zawiera kamerę i zintegrowane źródło światła bliskiej podczerwieni, które ma umożliwić obrazowanie MB jako fluoroforu – wzbudzona fala o długości 680 nm, wizualizacja przy około 710 nm. Kamera urządzenia została umieszczona około 20-30 cm od skóry pacjenta. Obszar ciała spływu chłonki z miejsca lokalizacji ogniska pierwotnego czerniaka badano za pomocą podręcznego systemu kamery gamma, Gamma Finder II (W.O.M World of Medicine GMBH; Berlin, Niemcy), przed wykonaniem nacięcia skóry, wraz z oceną widoczności MB przez skórę. Przez cały czas trwania zabiegu pole operacyjne było monitorowane kamerą NIR Quest Artemis. Podczas zabiegu chirurg miał możliwość obserwowania obrazu z kamery i operowania w czasie rzeczywistym. Ponadto węzły sprawdzono za pomocą kamery promieniowa gamma. Każdy węzeł widoczny w jednej z metod był traktowany jako wartownik. Jako tło do fluorescencji węzła wybrano otaczającą tkankę. Za pozytywny w NIRF uznano stosunek sygnału do tła (SBR - signal to background ratio)  $\geq 1,1$ . Podczas zabiegu kolorowy obraz pola operacyjnego był jednocześnie wizualizowany jako NIRF, aby ułatwić lokalizację węzła.

Wyniki badania przedstawiono w analizie statycznej, takiej jak mediana z wartościami minimalnymi i maksymalnymi oraz częstość wyrażona w procentach. Testy chi-kwadrat zostały użyte do sprawdzenia różnic między częstotliwościami obserwowanymi a częstotliwościami oczekiwanyymi w ramach hipotezy zerowej. Za istotną statystycznie uznano wartość  $p < 0,05$ . Wszystkie analizy statystyczne przeprowadzono

przy użyciu pakietu oprogramowania SPSS wersja 26.0 dla komputerów Mac (IBM Corp, Chicago, IL, USA).

## **Wyniki:**

### Publikacja 1

Wykorzystując właściwości fluorescencyjne błękitu metylenowego, można go stosować w pięciu głównych aspektach klinicznych. Wizualizacja moczowodów, identyfikacja przytarczyc, obrazowanie guzów trzustki, wykrywanie marginesów podczas wycięcia raka piersi oraz biopsja węzła wartowniczego raka piersi.

Wizualizacja śródoperacyjna moczowodu jest możliwa przy użyciu systemu NIRF oraz fluorescencji błękitu, szczególnie w procedurze laparoskopowej. Uszkodzenie moczowodu podczas zabiegu chirurgicznego jest stosunkowo rzadkim powikłaniem, ale wiąże się z poważnymi konsekwencjami klinicznymi. Błękit metylenowy wydalany z moczem po podaniu dożylnym umożliwia wizualizację moczowodu w NIRF. Dożylnie dawki MB mieszczą się w zakresie od 0,25 mg\kg do 1 mg\kg<sup>34,41</sup>. Fluorescencja moczowodu widoczna jest po 10 minutach od podania nawet do 60 minut. We wszystkich dawkach w przeprowadzonych badaniach wykazano znaczącą różnicę między średnim stosunkiem SBR we wszystkich punktach czasowych. Barenes i współpracownicy odnotowali najwyższy średni SBR przy dawce 0,75 mg/kg (średnia = 5,29, SD = 2,72, 95% CI 4,84–5,75), a najniższy przy 1 mg/kg (średnia = 3,66, SD = 1,89, 95% CI 3,37 –3,39)<sup>34</sup>. Ograniczeniem w wykorzystaniu MB w wizualizacji moczowodu może być dysfunkcja nerek i konwersja MB do postaci niefluorescencyjnej, co spowodowane jest redukcją i/lub zakwaszeniem środowiska.

MB od dawna jest stosowana w śródoperacyjnej identyfikacji przytarczyc przy zastosowaniu dawek od 3,5 do 7 mg/kg prowadząc do zmiany zabarwienia przytarczyc widocznych bez specjalnego sprzętu<sup>42,43</sup>. Tak wysokie dawki mogą wiązać się z występowaniem skutków ubocznych. NIRF umożliwia wizualizację przytarczyc przy niższych dawkach. Najniższa dawka błękitu metylenowego podawana dożylnie w celu odróżnienia przytarczyc od otaczającej tkanki wynosi 0,05 mg/kg, ale fluorescencja utrzymuje się tylko przez kilka sekund, co czyni ją mniej niż idealną do resekcji przytarczyc<sup>19</sup>. Dodatkowo przytarczyce wykazują autofluorescencję przy

długości fali od 750 nm do 785 nm. Przyczyna tego nie jest znana, może być związana z budową histologiczną i śródoperacyjnym ukrwieniem<sup>45,47</sup>.

Dożylna podaż MB powoduje również jego akumulację w komórkach guzów neuroendokrynnych<sup>48,49</sup>. Dzięki NIRF możliwa jest wizualizacja insulinoma o średnicy mniejszej niż 1 mm<sup>48,50</sup>. Fluorescencja MB może być wykorzystywana w śródoperacyjnej identyfikacji PNET trzustki. Zastosowanie fluorescencji MB w tym przypadku jest w trakcie dalszych badań.

Tummers i wsp. wykorzystali właściwości fluorescencyjne MB do oceny marginesów wyciętych raków piersi podczas operacji oszczędzającej<sup>24</sup>. Przedoperacyjne dożylnie podanie błękitu metylenowego w dawce 1 mg/kg pozwoliło na wizualizację marginesów wyciętych guzów za pomocą fluorescencji NIR.

Jiang i wsp. wykazali możliwość wykorzystania właściwości fluorescencji MB w identyfikacji węzła wartowniczego w raku piersi<sup>51</sup>.

## Publikacja 2

W przeprowadzonym badaniu radioaktywny technet był obecny we wszystkich przypadkach 20/20 (100%). U 3/20 (15%) pacjentów węzeł wartowniczy był widoczny przez skórę w NIRF. Mediana SBR dla wizualizacji węzła wartowniczego wyniosła 3,15 (zakres 2,7 do 3,5). U 4 pacjentów (20%), naczynia limfatyczne były widoczne w NIRF zanim były widoczne gołym okiem. Mediana SBR dla wizualizacji naczyń limfatycznych za pomocą fluorescencji wyniosła 3,69 (zakres 2,7 do 4,2). Węzły wartownicze były widoczne przez fluorescencję MB w 14 przypadkach (65%). Mediana SBR dla wizualizacji węzła wartowniczego podczas preparacji tkanki limfatycznej metodą fluorescencji wyniosła 2,49 (zakres od 1,5 do 5,7). Tylko dwóch pacjentów poddanych SLND miało przerzuty do tych węzłów, co wymagało całkowitej limfadenektomii podczas następnej operacji. U obu tych pacjentów węzeł wartowniczy wykazywał fluorescencję. U 16 pacjentów (80%) węzeł chłonny był zabarwiony na niebiesko, co pozwalało na jego identyfikację gołym okiem. U trzech z tych pacjentów węzeł wartowniczy był niebieski, ale nie zidentyfikowano fluorescencji. U jednego pacjenta węzeł chłonny wykazywał fluorescencję, ale gołym okiem nie było widoczne niebieskie zabarwienie. Spośród czterech pacjentów z widocznymi fluorescencyjnie kanałami limfatycznymi, tylko jeden węzeł wartowniczy

wybarwiono na niebiesko, ale nie wykryto fluorescencji. Przeanalizowano również związek między fluorescencją węzła wartowniczego a czynnikami, takimi jak płeć, lokalizacja czerniaka i owrzodzenie. Nie znaleziono istotnego związku między fluorescencją a tymi czynnikami. Podczas badania klinicznego nie wystąpiły żadne działania niepożądane związane ze stosowaniem błękitu metylenowego. Nie było również poważnych powikłań chirurgicznych.

### **Wnioski:**

#### Publikacja 1

Błękit metylenowy jest stosowany w medycynie od dziesięcioleci, zwłaszcza ze względu na jego dostępność, szeroki profil bezpieczeństwa i wszechstronność postępowania. Jego właściwości fluoresencyjne znajdują szerokie zastosowanie w różnych przypadkach śródoperacyjnych zwiększając dokładność identyfikacji struktur anatomicznych.

#### Publikacja 2

Po raz pierwszy w warunkach klinicznych opisano przydatność fluoresencyjnych właściwości błękitu metylenowego w biopsji wartowniczej u chorych na czerniaka. Oczekuje się, że przyszłe badania udowodnią jego potencjał kliniczny.

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## **LISTA PUBLIKACJI I WSKAŹNIKI BIBLIOMETRYCZNE**

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Publikacja 1

### **Methylene Blue-Current Knowledge, Fluorescent Properties, and Its Future Use.**

Tomasz Cwalinski, Wojciech Połom , Luigi Marano, Giandomenico Roviello, Alberto D'Angelo, Natalia Cwalina, Marcin Matuszewski, Franco Roviello, Janusz Jaskiewicz, Karol Połom

J Clin Med. 2020 : vol. 9, nr 11, art. ID 3538, s. 1-12, bibliogr. 86 poz., streszcz. ang.

doi: 10.3390/jcm9113538.

Publikacja 2

### **Fluorescence Imaging Using Methylene Blue Sentinel Lymph Node Biopsy in Melanoma**

Tomasz Cwalinski, Jarosław Skokowski, Wojciech Połom, Luigi Marano, Maciej Swierblewski, Kamil Drucis, Giandomenico Roviello, Natalia Cwalina, Leszek Kalinowski, Franco Roviello Karol Połom

Surgical Innovation 2022, Vol. 0(0) 1–8

DOI: 10.1177/15533506221074601

			Liczba		
rok	tytuł czasopisma	char. merytory.	prac	IF	MEiN
2020	J. Clin. Med.	PPP	1	4,242	140
2022	Surg. Innov.	ORG	1	2,058	70
		razem	2	6,300	210

ORG – praca oryginalna

PPP – praca poglądowa/przeglądowa

W dalszej części zamieszczam publikacje składające się na niniejszą rozprawę doktorską.



Review

# Methylene Blue—Current Knowledge, Fluorescent Properties, and Its Future Use

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Received: 25 September 2020; Accepted: 30 October 2020; Published: 2 November 2020



**Abstract:** Methylene blue is a fluorescent dye discovered in 1876 and has since been used in different scientific fields. Only recently has methylene blue been used for intraoperative fluorescent imaging. Here, the authors review the emerging role of methylene blue, not only as a dye used in clinical practice, but also as a fluorophore in a surgical setting. We discuss the promising potential of methylene blue together with the challenges and limitations among specific surgical techniques. A literature review of PubMed and Medline was conducted based on the historical, current and future usage of methylene blue within the field of medicine. We reviewed not only the current usage of methylene blue, but we also tried to grasp its' function as a fluorophore in five main domains. These domains include the near-infrared imaging visualization of ureters, parathyroid gland identification, pancreatic tumors imaging, detection of breast cancer tumor margins, as well as breast cancer sentinel node biopsy. Methylene blue is used in countless clinical procedures with a relatively low risk for patients. Usage of its fluorescent properties is still at an early stage and more pre-clinical, as well as clinical research, must be performed to fully understand its potentials and limitations.

**Keywords:** methylene blue; fluorescence; fluorophore

## 1. Introduction

Ever since German chemist Heinrich Caro first prepared methylene blue in 1876, it has been described as the first fully synthetic drug used in medicine [1]. Throughout many decades, a multitude of usages have been discovered amongst the various fields of science including clinical medicine and surgery [2,3].

Methylene blue is commonly used in medical practice, especially as a dye in microbiological staining. This function has now lent itself to the operating theatre, where methylene blue is often used as a marker and indicator in various surgical techniques [4–6]. Recently, it has been used for the detection of intestinal, enterovesical, and bronchopleural fistulas [7,8]. It has been also used as a drug for treating methaemoglobin-induced encephalopathy and often aids in treating dermatological diseases,

such as herpes labialis, eczema herpeticum, oral candidiasis, and cutaneous leishmaniasis [2,9–14]. Another field where methylene blue (MB) can be used is as an antidote in cyanide poisoning [14–16]. Historically, it was used in patients with urinary tract infection—as an antiseptic and stimulant of mucous surfaces, although it is no longer recommended in either [4,17]. Additionally, we can use MB intravenously in different doses to visualize enlarged parathyroid glands, observe the ureters and evaluate any potential damage [18,19]. Methylene blue (MB), also known as methylthioninium chloride, is a thiazine dye that can be used as a medication, and can be administered orally, subcutaneously or intravenously. The intravenous setting should be administered slowly—usually over 3 to 10 min [20,21]. Methylene blue is excreted in the urine 4 to 24 h after intravenous administration, with a half-life of 5 to 6.5 h [17]. Despite this, it is not necessary to reduce the dose in patients with renal insufficiency. However, it should be used with caution [14]. There is no specific dosage guidance for patients with liver failure [20].

Lastly, methylene blue can be used to detect lung nodules during thoracoscopic procedures [6]. Interestingly enough, some patients metabolize the methylene blue molecule into a non-fluorescent leucomethylene blue with an unclear impact on the patient [17]. Despite all of this, the most traditional use of methylene blue remains the detection of sentinel nodes in different cancers [22–27] as initially proposed by Giuliano et al. for breast cancer in 1994 [28].

The main effect of methylene blue on molecular level is through modulation of the cyclic guanosine monophosphate (cGMP) pathway. Additionally, multiple cellular and molecular targets of the MB compound have been identified. Many studies describe in detail the mechanisms of MB at the molecular level [19,29].

## 2. Fluorescence

Near-infrared (NIR) imaging is a novel technique that uses near-infrared light to help surgeons visualize different structures and allows them to see beyond the normal white light spectrum [30]. The NIR fluorescence visualization requires a fluorophore to work together with an imaging system to detect and measure its fluorescence after excitation. Depending on the clinical situation, fluorophores can be administered locally, but also systemically.

Fluorophores are excited by a specific wavelength of near-infrared light that reemits back an appropriate light visualization. The wavelength of this light is a longer one [31]. Due to this, near-infrared (NIR) light (700–900 nm) allows visibility of different structures about a 10 mm depth into the tissue, in the distinction of visible light [32–34]. Within the 700–900 nm spectrum, no autofluorescence is detected in tissues. The NIR fluorescence imaging requires the fluorophore coupled with an imagining system to show the fluorescence after excitation, then the compound detection is measured afterwards [32]. Many of the NIR fluorescence systems available on the market can alter the fluorescent signal with an overlaid color, which facilitates intraoperative orientation.

In most cases, lamps in the operating theatre have to be dimmed to the same level used during laparoscopic procedures [26].

The Federal Drug Administration (FDA) and the European Medicines Agency (EMA), have approved the clinical use of Indocyanine Green (ICG), Methylene Blue (MB) and 5-amino-levulinic acid (5-ALA) fluorophores. In our review, we focus on methylene blue, emphasizing that it is not considered a pure probe, due to its visual light emission spectrum of 400–700 nm [35]. Methylene blue has an excitation peak of about 700 nm, an excitation wavelength of 668 nm, plus an emission of 688 nm, which is visible to the naked eye [35,36]. Similar results were presented by other laboratories. Methylene blue presents with an absorption peak at 665 nm with a shoulder at 610 nm, presenting an additional peak at 293 nm [37]. It has a quantum yield of 0.52. Moreover, the emission spectra of MB is about 686 nm with a stoke shift of 21 nm. The clinical properties of MB are limited by its' hydrophobic nature. This is the reason why methylene blue presents less tissue penetration while at the same time, more autofluorescence of the background tissue. In comparison, the ICG fluorescence

spectrum is about 800 nm, with 5-ALA has a spectrum of about 510 nm, outside of the NIR fluorescence range [38,39].

### 3. Fluorescent Clinical Use

Methylene Blue when used together with a special NIR system for the detection of fluorescence, allows for the visualization of previously hidden structures during surgery. Below, we describe the major domains where the MB NIR guided surgical technique can be used.

#### 3.1. Visualization of Ureters

Iatrogenic ureter injury during surgery is a rare adverse event, but is considered one of the most serious complications. It is especially common during lower abdomen surgeries—especially gynecological interventions, where the prevalence ranges between 0.15 to 1.5% [36,40–42]. In colorectal procedures, the lower third of the left ureter is most prone to damage. [43]. Faster recognition of the ureter, although challenging in cases of previous surgery or radiotherapy of the pelvic floor, not only makes it easier to avoid potential damages, but it also helps in identifying different anatomical structures such as nerves and gonad vessels [44]. Intraoperative diagnosis of ureteral injury enables immediate repair and reduces negative consequences on patient's health [45] Intra-operative localization of the ureter often uses a light ureter catheter, particularly in laparoscopic surgery. However, this is an additional invasive procedure for patients, with an increased risk for further complications [46].

Verbeek et al. was the first to report the use of NIR fluorescence-guided surgery to visualize the ureters by intravenous injection of methylene blue [47]. In 12 patients, both ureters were detected 10 min after peripheral vein injection, with the signal lasting up to 60 min after administration. Three different doses of MB 0.25, 0.5, and 1 mg/kg were used. Statistically, these groups differed only in the time of exposure. Conversely, Al Taher et al. managed to visualize both ureters in only 5 out of 10 patients [48].

Barnes et al. reported identification of 64 out of 69 ureters during colorectal surgery of 40 patients [36]. Moreover 14 of ureters were not visible under white light. A group of 50 ureters were visible under both white and fluorescence light, but 14 of them were easier and faster to identify using the NIR camera. From the anatomical point of view, in 10 cases ureter was seen in a different localization than expected (14.5%). The authors divided patients into four groups based on MB concentrations used: 0.25, 0.5, 0.75, or 1 mg/kg. A significant difference has been shown between the mean signal-to-background ratio across all time points. The highest mean signal-to-background ratio was observed using the 0.75 mg/kg dose (mean = 5.29, SD = 2.72, 95% CI 4.84–5.75), and the lowest at 1 mg/kg (mean = 3.66, SD = 1.89, 95% CI 3.37–3.39). On the other hand, Yeung et al. showed that intra-operative concentrations of 1 mg/kg gave the strongest signal [49]. After administration of methylene blue intravenously during surgery, the highest fluorescence was obtained between 9 and 20 min after administration with a peak at 14.4 min. In all cases, the autofluorescence was low and the mean signal to the background ratio was 2.74. Figure 1 presents our experience in searching ureter during anterior resection of the rectum using Quest Spectrum camera, Quest, Netherlands (Figure 1).



**Figure 1.** Fluorescent visualization of a ureter during rectal cancer anterior resection.

One of the limitations of using methylene blue to visualize the ureters is the potential impairment of renal function because it is excreted by the kidneys. Another limitation is the restriction of MB use only for patients who are capable of converting MB into the non-fluorescing leucomethylene blue, which is caused by the reduction and/or acidity of the environment [17,50].

Allergic reactions to MB occur more frequently at doses above 5 mg/kg, therefore, use of methylene blue should be with the smallest efficacious dose [50–53]. With lower doses of MB used, it is still possible to visualize ureters with NIR fluorescence and reducing the risk of side effects at the same time [47]. The standard 1% concentration (i.e., 31.3 mmol/L) of MB used in clinical settings, such as that used during sentinel node biopsy, for instance, presents no fluorescence due to the quenching phenomenon [50]. The quenching threshold of MB diluted in urine is less than 20 µmol/L. In higher concentrations, moderately high NIR fluorescence is visible, with peak excitation at 668 nm, extinction coefficient of  $69,100 \text{ mol/L}^{-1} \times \text{cm}^{-1}$ , peak emission at 688 nm [50].

When diuretics are used in conjunction with methylene blue, the increase in urinary excretion does not affect the fluorescence of the MB dye. This could be related to the greater dilution of urinary-methylene blue [50]. Matsui et al. assumed that this phenomenon is caused by furosemide, which affects the conversion of methylene blue into non-fluorescent leucomethylene blue [50].

Novel experimental dyes are currently in development [50]. In the review by Slooter et al., eight experimental dyes that could be used to identify ureters were described [54]. Two of the proposed dyes present interesting characteristics: CW 800–BK and ZW 800–1. Both are currently being investigated in ongoing clinical trials and the results of those trials should be available soon. The remaining six dyes are still in the earlier stages of research: CW 800–CA, Fluorescein, Liposomal ICG, Genhance 750, UL–766, Ureter glow.

### 3.2. Thyroid and Parathyroid Glands

Localization and dissection of the parathyroid glands is still a formidable challenge during surgery. Detection of the enlarged glands is often difficult due to their variability in number and location. Incorrect diagnosis of the parathyroid glands during surgery may result in serious complications [55]. The first report of using methylene blue to visualize the parathyroid glands dates back to 1971, when after injecting a high dose of MB (5 mg/kg) intravenously, surgeons noticed that the parathyroid glands gradually changed in color (blue) for one hour, followed by a gradual change back to normal after two and a half hours [56]. Moreover, the normal parathyroid gland tissue was stained blue only at the periphery of the gland, while the adenomas completely changed color. Despite these results, there are currently no prospective clinical trials using high doses of methylene blue during surgery on the parathyroid glands [57]. This is most likely due to the increased risk of side effects associated with the higher dose of MB [57].

Currently, there are nine parathyroid identification methods available on the market: autofluorescence spectroscopy, autofluorescence imaging, ICG imaging, methylene blue fluorescence imaging, 5-ALA, optical coherence tomography, laser speckle contrast imaging, dynamic optical contrast imaging, and Raman spectroscopy [35,58–65].

Methylene blue is traditionally injected intravenously in a high dose (3–7.5 mg/kg) to allow for the naked eye to be able to see the enlarged parathyroid glands as they are stained blue, but as mentioned earlier, these doses are associated with many adverse effects and should be used with caution [66,67]. The NIR fluorescence technique makes it possible to detect the glands by using lower doses of MB. According to Hillary et al. the optimal dose for this technique is 0.4 mg/kg, which makes it possible to distinguish the parathyroid glands from the surrounding tissue for a reasonable amount of time [26]. However, directly after intravenous administration of this MB dose, there can be a temporary false decrease in hemoglobin saturation, with values reading as low as 65%. This drop disappears after about 30 s, and is due to a temporary impaired reading of the pulse oximeter—a disorder of light absorption in the blood [68]. There are no hemodynamic changes observed, and the oxygen saturation readings quickly return back to baseline [26].

The lowest dose of methylene blue that has been administered intravenously to differentiate parathyroid glands from the surrounding thyroid tissue is 0.05 mg/kg, but the fluorescence only lasts for a few seconds, which makes it less than ideal for resections of the parathyroid glands [26].

Fluorescence of an abnormal parathyroid gland is greater than a normal one [26]. However, the Mc Wade et al. study shows that there is no significant difference of near infrared fluorescence visualization between a healthy parathyroid gland (signal-to-background ratio of  $4.51 \pm 1.24$ ) and an abnormal one ( $4.81 \pm 0.80$ ) [55]. During the injection of higher doses of MB, the fluorescence of abnormal parathyroid glands increases more compared to the surrounding tissue, especially to that of normal parathyroid glands [26].

Surprisingly, we can observe auto-fluorescence of parathyroid glands prior to fluorophore injection [26]. De Leeuw et al. [58], Mc Wade et al. [55], and Paras et al. [69] all mention this in their studies, where they detected the parathyroid glands by using their autofluorescence at wavelengths of 750 nm and 785 nm, which is 2–11 times more visible than the thyroid and surrounding structures [55,58,69]. Hillary et al. demonstrated the same phenomenon by using a wavelength of 680 nm [26]. The cause of parathyroid hyper-fluorescence is unknown and seems to be independent from its' histological structure and intraoperative blood supply [58,70].

In search for parathyroid adenomas, Van der Vorst et al. administered 0.5 mg/kg of MB (at a concentration of 10 mg/mL) intravenously for 5 min to a group of 10 patients after dissecting the neck tissues [71]. This resulted in 9 out of 10 adenomas detected using the NIR methylene blue imaging method during the operation. Furthermore, only 7 out of 9 patients had a positive preoperative  $^{99m}$ Tc-sestamini single photon emission computed tomography scans and in 2 of the patients the adenomas were only seen with the NIR MB visualization [71].

### 3.3. Pancreatic Neuroendocrine Tumor

Methylene Blue accumulates in neuroendocrine tumors when injected intravenously, but the precise mechanism of this action is unknown. Initial reports of its clinical application have been described [72].

The first neoplastic lesion of the pancreas (insulinoma) to be stained by using high doses (5 mg/kg) of MB was seen in 1974 [73]. Winner et al. showed that by using MB as a fluorescent dye, it can be useful in visualizing insulinoma in NIR light, in an animal model [72]. When intravenously injecting MB, the NIR fluorescence from the pancreas remains relatively at the same level of a 3.0 signal to background ratio for about 60 min. Depending on the usage of MB, a different signal to background ratio can be achieved. A higher ratio is achieved when the MB administration occurs in a rapid bolus (5–20 s), when compared to a slow infusion (15–20 min). However, both techniques of administering MB displayed contrast within the pancreas. The animal models (pigs) were divided into individual groups of test subjects and received different doses of methylene blue—0.25, 0.5, 1, and 2 mg/kg. The signal to background ratio (SBR) was  $\geq 1$  in doses greater than 1 mg/kg, and was statistically significant with the baseline ( $p < 0.05$ ). The lack of statistical difference in SBR doses of 1 and 2 mg/kg may be associated with a plateau phase of the signal strength, thus reaching the quenching threshold or saturation of the signal absorption process. Neoplastic lesions (insulinoma) of the pancreas were able to be distinguished from healthy tissue in NIR fluorescence within 2 min after intravenous injection of MB. Furthermore, NIR fluorescence was able to detect neoplastic lesions of the pancreas with a diameter less than 1 mm. The higher uptake of MB in the neoplastic tissue showed an insulinoma to pancreas ratio of  $3.7 \pm 0.5$ .

Preoperative and intraoperative difficulties of visualization of the pancreatic neuroendocrine tumors (PNET) may lead to incomplete resection, but in the case of suspected PNET associated multiple endocrine neoplasia type 1 and 2, surgeons have been able to use NIR imaging during surgery to identify lesions [74]. In one particular case, tumors were identified using preoperative magnetic resonance imaging studies. Only four lesions were visible during positron emission tomography (PET) scans. Using near-infrared imaging with MB allowed for visualization of the neuroendocrine lesions intraoperatively, with more than 20 fluorescent lesions detected in the entire pancreas. This discovery led to a change in the surgical approach and a total pancreatectomy was performed.

Pancreatic solitary fibrous tumors have also been detected using MB as a fluorescent dye with a 1 mg/kg dose, resulting in a SBR of about 3, which remained stable for nearly 15 min [75]. As a result, it was possible to localize a tumor in the uncinate process of the pancreas by using NIR fluorescence.

The possibility of using MB in NIR visualizations of paraganglioma with a smaller second lesion not seen by the naked eye has also been identified [76].

### 3.4. Breast

Intraoperative localization of breast cancer tumors is challenging and the high rate of positive margin resections is still a vast problem [77]. Therefore, intraoperative techniques capable of visualizing cancer cells are needed. Based on an analysis of different studies, a total of 402,357 patients diagnosed with breast cancer (invasive and ductal carcinoma *in situ*) were identified, with a mean re-operation rate (re-excision or mastectomy) of 27.49%, varying between treatment centers [78].

An interesting application of methylene blue and its fluorescent properties is its role in breast cancer margin detection during breast-conserving treatment, as proposed by Tummers et al. [79] using NIR fluorescence imaging, they detected tumors of the breast in 20 of 24 cases [40]. Patients were divided into two groups: those with early imaging, with methylene blue administered intravenously 5 min before the start of the operation, and those with delayed imaging, with methylene blue administered 3 h prior to the surgery. All patients received a 1 mg/kg dose of MB. [79]. There was no statistical difference between the groups when analyzing the tumor to background ratio. Among these patients, four had positive surgical margins. In two cases with the use of NIR fluorescence, a positive margin was visible on the surface of the excised tissue in the post-operative bed and also in the cut margin. In one of the cases, by using MB fluorescence, a second surgery was avoided. In one patient, the tumor was not visible using the NIR fluorescence. The authors suggested that this may have been the result of the transformation of methylene blue into a non-fluorescent form. Another negative case was the result of logistical problems during the study. There were no statistical changes in the histopathological type of breast cancer ( $p = 0.22$ ).

Another study by Zhang et al. showed the possibility of using MB fluorescence imaging to identify breast cancer [59]. This study involved 30 breast cancer patients divided into two groups—patients who received pre-operative chemotherapy, and patients without neoadjuvant treatment. All patients received a 1 mg/mL dose of methylene blue intravenously 3 h before their surgery. To aid in fluorescence evaluation, a camera was used that had been prepared for methylene blue visualization. In 16 out of 20 patients (80%) who did not receive pre-operative chemotherapy, the dissected breast specimens showed a fluorescent contrast (signal-to-background ratio: 1.94 + 0.71). In the group of patients that received neoadjuvant chemotherapy fluorescence of the tumor could be observed in 3 of 10 cases (30%), suggesting that neoadjuvant chemotherapy has an impact on the imaging result ( $p < 0.05$ ) and that the detection of breast cancer using MB fluorescent contrast was strongly affected. It is worth mentioning that in 5 cases, the fluorescence showed additional tissue that was excised in the group of patients without neoadjuvant chemotherapy. During the histopathological examination, this additional tissue turned out to be benign hemorrhagic tissue. The statistical analysis of the 35 samples showed a sensitivity of 0.63 and a positive predictive value of 0.79 by using MB-based NIR fluorescence.

In the paper by Jiang et al., the authors presented the first cases of sentinel node biopsy using methylene blue together with fluorescence imagining [60]. These promising videos showed potential benefits on the identification of lymph vessels, location of sentinel nodes, and the patterns of breast lymphatic flow. Future studies on this topic are needed to show its real potential as well as limitations.

## 4. Side Effects

Methylene blue is a safe drug if used within therapeutic doses of <2 mg/kg [35]. Patients can experience slight pain after intravenous injections, which resolves after flushing the access site with saline [59,79]. Other adverse symptoms include a burning sensation, rash, abscess, necrosis, and even ulceration [4]. Green colored urine can even be observed after administration of MB [4].

Intravenous injection of MB causes a temporary desaturation in blood, which can be measured when using pulse oximetry, but oxygen levels return to a normal shortly thereafter [61,62,68]. High intravenous doses of methylene blue can cause nausea, vomiting, abdominal pain, precordial pain, dizziness, headache, profuse sweating, dyspnea, hypertension, and mental confusion [4,17,20]. With subcutaneous and intradermal administration, adverse skin reactions, such as erythematous macular lesions, superficial ulcers, and abscess formation may form [63,64]. In higher systemic doses, toxic reactions may occur, such as cardiac arrhythmias, coronary vasoconstriction, lower cardiac output, decreased renal function, as well as decreased mesenteric blood flow. Pulmonary vascular pressure can increase together with pulmonary vascular resistance and impaired gas exchange can be observed [35,62].

Localized skin and fat necrosis have been reported after subcutaneous injection during sentinel lymph node biopsy [63]. Tattoo persistence has also been observed up to 1 year after the operation, as well as prolonged postoperative radiotherapy and breast edema [65]. Severe immunoglobulin hypersensitive reaction has been reported in 1% MB when used for tubal permeability during general anesthesia [80,81]. According to the review by Bezu et al. [80], anaphylactic reaction to methylene blue dye during sentinel node biopsy is extremely rare. The prevalence of reactions to other blue dyes, namely isosulfan blue and patent blue V, varies between 0.07% and 2.7% [80].

Serotonin syndrome may occur after using methylene blue, especially if used together with other serotonergic drugs like selective serotonin reuptake inhibitor (SSRIs) and serotonin-norepinephrine reuptake inhibitor (SNRIs) drugs. Most cases of serotonin syndrome occurred after doses of 1–8 mg/kg MB via intravenous injection used when trying to visualize the parathyroid glands [34,82] in patients taking these medications. As such, the use of methylene blue should be avoided in these patients [10,20,82].

Methylene blue is contraindicated in patients who experienced hypersensitivity reaction and in patients with renal insufficiency because it is excreted through the kidneys [83]. It is also contraindicated in glucose-6-phosphate dehydrogenase (G6PD) deficiency and in patients with Heinz body anemia [84].

Methylene blue is potentially teratogenic and should not be used during pregnancy [4,20,85,86].

## 5. Conclusions

Methylene blue has been used in the field of medicine for decades, due to its accessibility, wide safety profile, and proved versatility. Despite its limitations, it has shown tremendous promise when used as a NIR fluorophore, especially thanks to its fluorescent properties. The field of near-infrared imagining with methylene blue as a fluorophore is an interesting surgical technique that demands further clinical investigation.

**Author Contributions:** Conceptualization, T.C., W.P., L.M., G.R., A.D., N.C., M.M., F.R., J.J. and K.P.; methodology, T.C., L.M., G.R., A.D., N.C. and K.P.; software, T.C., W.P., L.M., G.R., A.D. and N.C.; validation, T.C., M.M., F.R., J.J. and K.P.; formal analysis, T.C., W.P., L.M., G.R., A.D., N.C. and K.P.; investigation, T.C. and K.P.; resources, T.C. and K.P.; data curation, T.C., W.P., L.M., G.R., A.D., N.C., M.M., F.R., J.J. and K.P.; writing—original draft preparation, T.C., W.P., L.M., G.R., A.D., N.C., M.M., F.R., J.J. and K.P.; writing—review and editing, T.C., W.P., L.M., G.R., A.D., N.C., M.M., F.R., J.J. and K.P.; visualization, T.C., W.P., L.M., G.R., A.D., N.C., M.M., F.R., J.J. and K.P.; supervision, M.M., F.R., J.J. and K.P.; project administration, T.C. and K.P. All authors have read and agreed to the published version of the manuscript.

**Funding:** The authors have not declared a specific grant for this research from any funding agency in the public, commercial, or not-for-profit sectors.

**Acknowledgments:** All authors contributed to all stages of this work.

**Conflicts of Interest:** The authors declare that there is no conflict of interest

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# Fluorescence Imaging Using Methylene Blue Sentinel Lymph Node Biopsy in Melanoma

Surgical Innovation  
2022, Vol. 0(0) 1–8  
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DOI: [10.1177/15533506221074601](https://doi.org/10.1177/15533506221074601)  
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## Abstract

**Introduction:** Fluorescence imaging of sentinel node biopsy in melanoma is a novel method. Both indocyanine green (ICG) and methylene blue (MB) have fluorescent properties. The aim of this study was to present, for the first time in a clinical series of patients, the possible usage of MB as a fluorescent dye for sentinel node biopsy during surgery for melanoma.

**Material and methods:** Twenty patients with skin melanoma, who were candidates for sentinel node biopsy were enrolled in our study. All patients underwent simultaneous use of standard nanocolloid and blue dye. Transcutaneous visualization of the sentinel node, visualization of lymphatic channels as well as sentinel node fluorescent visualization were all measured. We also performed calculations of Signal to Background ratios (SBR).

**Results:** In 15% (3/20) of patients, the fluorescent sentinel node was visible through the skin. The median SBR for the sentinel node visualization by fluorescence was 3.15 (range, 2.7–3.5). Lymphatic channels were visible in lymphatic tissue via fluorescence before visualization by the naked eye in 4 patients (20%). The median SBR ratio was 3.69 (range, 2.7–4.2). Sentinel nodes were visible by fluorescence in 13 cases (65%). The median SBR ratio was 2.49 (range, 1.5–5.7). No factors were found to be associated with fluorescent MB visualization of a sentinel node during biopsy.

**Conclusion:** This is the first clinical study presenting the usefulness of fluorescent sentinel node biopsy in melanoma patients using MB as a fluorophore. Further studies are necessary to provide methods for its' clinical implementation.

## Keywords

image guided surgery, surgical oncology, surgical education

## Introduction

Melanoma is a malignant tumor of the skin that has exhibited a significant increase in incidence in the last few decades worldwide.<sup>1</sup> Accurate determination of the stage of the disease is key to determining the correct plan of treatment. One of the main evaluation elements, apart from the depth of infiltration of the primary lesion, is the regional lymph node status.<sup>2</sup> Regional lymph nodes should be evaluated for metastasis prior to initiating melanoma treatment.<sup>2</sup> The patient's lymph node status is the single most important prognostic factor for those patients who present with early-stage cutaneous melanoma.<sup>3</sup> The biopsy of sentinel nodes in clinically non-suspect lymph nodes is routinely performed to assess disease advancement.<sup>4</sup> Micrometastases can occur in up to 25% of patients with clinically negative lymph nodes.<sup>5</sup>

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Morton was one of the first to describe the procedure of sentinel node identification using blue dye in 1992.<sup>5</sup> In 1993, the use of radiotracers to detect the sentinel node in melanoma was described in the literature.<sup>6</sup> Three years later, Albertini et al. combined both methods—the use of blue dye and radiotracers—which increased the sensitivity of visualization of sentinel nodes.<sup>7</sup> Currently, the combination of both methods is frequently used in cancer treatment centers worldwide.<sup>7</sup> With the simultaneous use of technetium-99m (99mTC) and blue dye, the frequency of correct identification of the sentinel node location is 96–99%.<sup>8</sup> Apart from methylene blue (MB) and radioactive colloids, other dyes that can be used to visualize sentinel nodes include indocyanine green (ICG), which also has fluorescent properties. A new age of image-guided surgery began in 2005, when Kitai et al. presented the first clinical description of ICG as a fluorophore for sentinel node biopsy in breast cancer using near-infrared light.<sup>9</sup>

Interestingly, MB also presents with fluorescent properties and could potentially be used as a fluorophore.<sup>10,11</sup> Methylene blue works as a fluorophore when excited by lights of 680 nm and can be visualized at approximately 710 nm.<sup>10</sup> Methylene blue is currently used as a fluorescent dye in the visualization of parathyroid glands during surgery, the localization of the ureters to prevent accidental damage during operations, and the localization of different pancreatic tumors, as well as in breast cancer margin detection during breast conserving treatment.<sup>11–18</sup> Other fluorophores, such as fluorescein, are under investigation for their possible use in fluorescence-guided sentinel node biopsy in melanoma patients.<sup>19</sup>

However, ICG is not registered in some countries as a standard dye for sentinel node biopsy in melanoma patients still new fluorophores are searched for image-guided navigation. Moreover, a revolution in camera systems began and a camera system that visualizes not only ICG but also methylene blue using its fluorescent properties is available.

The aim of this study was to present, for the first time in a clinical series of patients, the possible usage of MB as a fluorescent dye for sentinel node biopsy during melanoma surgery.

## Methods

Twenty patients with melanoma of the skin on the trunk or extremities were prospectively enrolled in this study. This study was approved by the institutional review board (no. NKBBN/99/2018). All patients signed an informed consent form. Every patient included in the study had indications for sentinel node biopsy in accordance with current guidelines.<sup>20</sup>

All subjects were injected intradermally with <sup>99m</sup>TC prior to surgery (1–3 h before operation) and with MB (MB) dye

in the operating room. Up to 1 mL of MB (Metiblo 10 mg/mL, Laboratories Sterop NV, Brussels, Belgium) was injected around the scar. The MB was injected into the dermis separately after induction of anesthesia and prior to prepping the patient. There were no adverse reactions to the MB.

Intraoperative near-infrared imaging was performed using a handheld device, NIR Quest Artemis (Quest Artemis, Netherlands). This device includes a camera and an integrated near-infrared light source intended to aid in the imaging of MB as a fluorophore when excited by the wavelength of 680 nm, with visualization at approximately 710 nm. The camera was installed on the flexible arm, and fluorescence imaging was performed at a distance of about 20–30 cm from the surgical field. Each lymph node basin was examined with a handheld gamma camera system, Gamma Finder II (W.O.M. World Of Medicine GMBH; Berlin, Germany), prior to making a skin incision, along with assessment of the visibility of MB through the skin (yes or no). From the initial incision of the skin until the sentinel lymph node was localized, the image was continuously monitored using the NIR Quest Artemis camera. While using the camera, it was necessary to darken the operating room lights to avoid any visual disturbances. Similar to laparoscopic procedures, the surgeon was able to watch the image and operate at the same time. The possibility of the camera system to visualize fluorescence images while “hands free” enabled continuous visualization of the lymphatic vessels and nodes during surgery. Further, the nodes were inspected for radiation markers. Each node that was visible in one of the methods was treated as sentinel. Whether an sentinel lymph node (SLN) was fluorescent depended on the Signal to Background ratio (SBR). A region of interest (ROI) from adjacent tissue was selected as a background. A SBR  $\geq$  1.1 with NIR fluorescence was considered positive. The Quest Artemis (Quest Medical Imaging, Middenmeer, The Netherlands) is designed and developed for open and minimally invasive image-guided surgery using near-infrared fluorescence imaging (NIRF). The fluorescence imaging system is designed to visualize two types of fluorescent probes: Cy5.5 and ICG, or any other probe that has similar fluorescence properties that are not visible to the naked eye. For this study, the Cy5.5 mode was used to visualize the MB. Methylene blue in tissue is illuminated with light at a wavelength of 680 nm and visualized at approximately 710 nm. During imaging, a color image of the surgical field was visualized simultaneously as the NIRF to assist in surgical guidance.

## Statistical Analysis

Descriptive statistics were reported as median, with minimum and maximum values or frequency with percentage. Chi-squared tests were used to test for differences

**Table 1.** Clinico-pathological Characteristics of 20 Enrolled Patients.

Variable	Category	No Cases (%)
All patients		20 (100)
Age, years [median (min, max)]		60 (21, 85)
Sex		
	Female	10 (50)
	Male	10 (50)
BMI [mean (min, max)]		27.05 (19, 35)
Location of primary		
	Head and neck	0 (0)
	Trunk	8 (40)
	Upper extremity	3 (15)
	Lower extremity	9 (45)
Laterality		
	Right	11 (55)
	Left	7 (35)
	Bilateral/midline	2 (10)

**Table 2.** Histopathologic Features of Enrolled Patients.

Variable	Category	No. Cases (Percentage)
Type of melanoma		
	Superficial spreading	15 (75)
	Lentigo maligna melanoma	2 (10)
	Nevoid	3 (15)
Breslow, mm [mean (min, max)]		[1.6 (.6, 5)]
Ulceration		
	No	18 (90)
	Yes	2 (10)
Mitotic rate [mean (min, max)]		[1.6 (0, 9)]
pT stage		
	T1	4 (20)
	T2	16 (80)
	T3	16 (80)
	T4	4 (20)
pN stage		
	N0	18 (90)
	N+	2 (10)

between observed frequencies and frequencies that were expected under the null hypothesis. A *P*-value < .05 was considered statistically significant. All statistical analyses were performed using the SPSS version 26.0 software package for Mac (IBM Corp, Chicago, IL, USA).

## Results

The study enrolled 20 patients—10 women and 10 men as clinical subjects—all of whom underwent SLN mapping with both radioactive tracer and MB using standard naked eye visualization and fluorescence guidance. The median age of the subjects was 60 years (range, 21–85 years). A list of the patients' clinicopathological characteristics is provided in Table 1. Anatomic locations of primary melanoma sites were distributed accordingly: 8, trunk; 3, upper extremity; and 9, lower extremity. Histopathological data are listed in Table 2. Melanoma advancement was in the range between pT1a and pT3b (patients with T1a were enrolled in the study if they had additional risk factors such as ulceration or mitoses  $\geq 1/\text{mm}^2$ ).

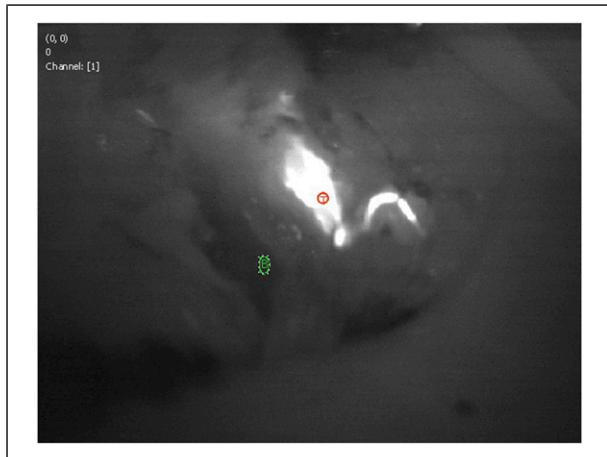
In all cases, Tc-99m uptake was present. In 15% (3/20) of patients, the sentinel node was visible through the skin by NIR fluorescence (Table 3). The median SBR for the sentinel node visualization by fluorescence was 3.15 (range, 2.7–3.5). Lymphatic channels were visible in lymphatic tissue via fluorescence before visualization by the naked eye in four patients (20%). The median SBR ratio for lymphatic channel visualization by fluorescence was 3.69 (range, 2.7–4.2). Sentinel nodes were visible by fluorescence with MB in 13 cases (65%) (Figure 1). The median SBR ratio for sentinel node visualization during lymphatic tissue preparation by fluorescence was 2.49 (range, 1.5–5.7). From all the recorded sentinel nodes, only in two patients were metastases identified that required lymphadenectomy as a second operation. In both of these patients, the sentinel node was fluorescent.

The sentinel node was identified by the naked eye when stained blue in 16 patients (80%). In three of these patients, the sentinel node was blue, but no fluorescence was identified. In one patient, the node was fluorescent but was not visible as blue by the naked eye. Out of the four

**Table 3.** Analysis of Sentinel Lymph Node Data.

Variable	Category	No. Cases (Percentage)
Radiocolloid nodes stained	No	0
	Yes	20 (100)
NIRF visibility through skin	Negative	17 (85)
	Positive	3 (15)
NIRF visibility of nodes	Negative	7 (35)
	Positive	13 (65)
Blue nodes stained (eye)	Negative	4 (20)
	Positive	16 (80)
NIRF visibility of lymphatic vessels	No	16 (80)
	Yes	4 (20)

NIRF: near-infrared fluorescence.



**Figure 1.** Sentinel node whit lymphatic vessels visible in near infra-red fluorescents using methylene blue.

patients presenting with fluorescent lymphatic channels, only in one case was the sentinel node stained blue but did not reveal fluorescence. We also performed analyses to assess the differences between observed frequencies and frequencies that were expected under the null hypothesis among the transcutaneous sentinel visualizations, as well as to identify the relationship between sentinel node fluorescence and factors such as patient's sex, localization of melanoma, laterality, and ulceration. No significant association was found between fluorescence and these factors (Table 4). No adverse events related to the use of MB occurred in this study. No major surgical complications were noted, and there were no operative fatalities.

## Discussion

The primary endpoint of this study was the detection rate of fluorescent lymph nodes during melanoma sentinel node procedures. In our study, only 65% of subjects exhibited fluorescence of sentinel nodes stained by MB. In four patients, the node was visible only by the naked eye,

and in one patient, by fluorescent camera only. Due to the limited research on the fluorescent use of MB, we had to compare this data with that of other fluorescent dyes, namely, ICG. According to a systematic review by Niebling et al., in melanoma, the identification rate for sentinel node biopsy using MB alone was 84%, for radiocolloid alone 99%, and a combination of radiocolloid and MB was 98%. The authors also analyzed the impact of fluorescent dye on the identification rate in sentinel node biopsy for melanoma.<sup>4</sup> They discovered that using ICG alone yielded a 100% identification rate, and a combination of radiocolloid and ICG also yielded an identification rate of 100%.<sup>4</sup> Here, we need to point out that MB and ICG are different fluorophores. There are a few technical issues that have to be analyzed that may explain the low rate of fluorescent sentinel node identification when using MB. First, both fluorophores are excited and detected by different wavelengths of light.<sup>11,21,22</sup> Moreover, MB presents with a lower tissue penetration capacity and a higher rate of autofluorescence. Methylene blue's depth of detection through tissue is also much lower—less than 1 cm, compared with ICG, which can penetrate about 1.5 cm.<sup>23</sup> Future research should thus focus on other clinical advantages of the dye for patients, such as safety, accuracy, and patients' satisfaction.

In the initial reports describing the use of ICG as a fluorophore, the authors pointed out that the possibly lower identification rate could be due to the quenching effect.<sup>24</sup> This effect is associated with a reduction of fluorescence emission as the concentration of the dye increases.<sup>25</sup> This can explain the lower rate of fluorescent sentinel nodes in our study, as we used a standard concentration of MB that is widely available on the market. Mieog et al. recommended a standard dose of .62 mg of ICG to express optimal fluorescence, thus decreasing the effect of quenching.<sup>25,26</sup> However, in breast sentinel node studies, various doses between .625-15 mg in a volume range of 1-5 mL revealed similar high detection rates.<sup>27</sup> Clearly, future studies are necessary to eliminate the

**Table 4.** Critical Values of the Chi-Square Test for Factors Associated with Near Infrared Fluorescence Visibility Thorough Skin and Sentinel Lymph Nodes.

Variable	SkinNIRF		Univariate Analysis (P value)		NodeCameraNIRF		Univariate Analysis (P Value)	
	0	1	0	1	0	1	0	1
Gender	1	8	.392	.220	4	6	6	.220
	2	9			3	7	7	
Localization	Head and neck	0	0	2.57	0	0	0	.982
	Trunk	8	0		3	5	5	
	Upper extremity	2	1		1	2	2	
	Lower extremity	7	2		3	6	6	
Laterality	Right	9	2	.443	4	7	7	.334
	Left	6	1		2	5	5	
	Bilateral/midline	2	0		1	1	1	
Ulceration	Yes	2	0	.392	1	1	1	.220
	No	15	3		6	12	12	

NIRF: near-infrared fluorescence.

problem of quenching in fluorescent sentinel node biopsy using MB. We hypothesize here that an optimal concentration of MB may increase the detection rate and help in a wider usage of its fluorescent properties.

Methylene blue is a common dye used in various medical applications. Due to its properties, it is not a pure fluorophore like ICG, but it can be used as a dye with fluorescent properties, that is, as a near-infrared tracer. Methylene blue has been used to locate sentinel nodes with the naked eye for several years. However, the use of its fluorescent properties in the location of the sentinel node is a new application. By using the NIR camera, we may be able to determine the course of the lymphatic pathway and the location of the sentinel node, which permits real-time transcutaneous and intraoperative visualization. The location of the sentinel node in melanoma is very variable and depends on the original site of melanoma.

An important problem to address with fluorophores used in sentinel node biopsy in melanoma patients is that in patients with localization of skin changes on the trunk, lymphatic drainage is complex and can drain to different regional stations. In our study, transcutaneous visualization of lymph nodes was possible in three patients (15%). The transdermal detection of sentinel nodes using ICG's fluorescent properties varies from 10% to 63.4% of patients.<sup>28,29</sup> Transcutaneous visualization of lymphatic vessels was possible in four (20%) of our patients. This finding is of special interest, especially in patients who are not good candidates for SPECT-CT. Here, we also have to emphasize the fact that lymphatic channel identification with fluorescence may improve the nodal extirpation as well as minimize damage to other lymphatic channels nearby, preventing further damage to lymphatic structures. This could lower postoperative complications associated with lymph collection. Future evaluations should include a group of patients with sentinel nodes visible only with fluorescence and not by naked eye. In our study, there was only one such patient (5%), which presents an avenue for further investigation. If the sentinel nodes are not visible by the naked eye, we may be able to increase the sensitivity of MB by adding fluorescence.

The most important advantage of MB is its safety profile as well as the history of its clinical usage worldwide.<sup>30,31</sup> Methylene blue's fluorescent properties have been used in many clinical situations, especially in the assessment of the parathyroid glands,<sup>32</sup> visualization of the ureters,<sup>11</sup> in breast cancer margin detection,<sup>13</sup> and pancreatic neuroendocrine tumor identification. Methylene blue is a safe drug that has been used in surgery for decades. However, severe immunoglobulin hypersensitive reactions to 1% MB used for tubal permeability during general anesthesia have been reported.<sup>33</sup> Desaturations may occur shortly after intravenous injection of MB, but oxygen saturation levels return to normal within seconds. Further, green discoloration of the urine can be

observed with the use of the dye. Bezu et al. reported no cases of an anaphylactic reaction after MB usage.<sup>34</sup>

Notably, the camera system that we used in this study could work for two separate dyes, namely, ICG and MB. This offers an opportunity to explore the use of both fluorophores for more applications. The price for such a system is comparable or just slightly higher than that of other similar ICG-fluorescence-only devices available worldwide. Further, the price for 1 mL of 10 mg/mL MB vial is about 2 euros, and 1 vial of ICG 25 mg costs about 75 euros.

In conclusion, this is the first report of fluorescent guided navigation surgery for sentinel node biopsy in melanoma patients. Fluorescence lymphography using the gold standard of the radiocolloid method together with MB, which can be detected not only by naked eye but also with fluorescent camera systems, might prove to be an attractive option for improvement in surgical navigation. Here, we have to point that results we obtained are far from gold standard detection. Despite its promise, there are still some issues that need to be addressed in the use of MB as a fluorophore. First, the problem of the quenching effect that is probably responsible for a smaller rate of detection should not be ignored. Another important limitation is the necessity of turning off the operating room lights during the operation, as shadowless lights that are used in an operating theater contain near-infrared light that can interfere with the fluorescent camera system. Further investigation regarding improvements to camera technology is necessary. The low penetration of fluorescent detection is another limitation of the wider clinical application of MB. Future studies will need to analyze the impact of high BMI on this issue. There is also the need to use a special camera system that is designed only for MB fluorescence detection; however, such a system is already available on market. Methylene blue as a gold standard dye in sentinel node biopsy presents us with new physical possibilities that allow us to visualize more with the use of new technologies. Further development and investigation are necessary to improve and standardize its usage in the field of image-guided surgery.

### Authors Note

This is the first clinical study presenting the usefulness of fluorescent sentinel node biopsy in melanoma patients using MB as a fluorophore. Methylene blue as a gold standard dye used in sentinel node biopsy presents with new physical possibilities that allow us to see more with the use of new technologies. Further development and investigation is necessary to improve and standardize its' usage in the field of image-guided surgery.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

## Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by specific grant for this research nr 02-0013/07/114 Medical University of Gdańsk.

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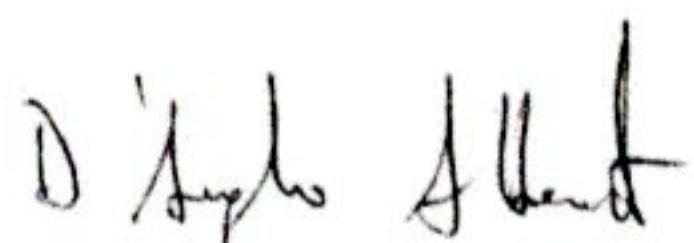
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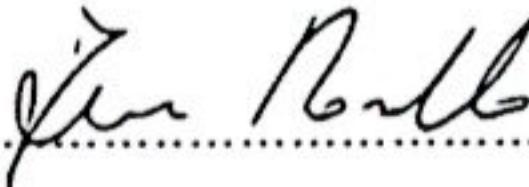
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Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek. Tomasza Cwalińskiego przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

KIEROWNIK  
Katedry i Kliniki Chirurgii Onkologicznej  
Gdańskiego Uniwersytetu Medycznego

prof. dr hab. n. med. Janusz Jaśkiewicz.....  
(podpis współautora)

Gdańsk, dnia 18.02.2022

Dr n. med. Jarosław Skokowski  
(tytuł zawodowy, imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. „Fluorescence Imaging Using Methylene Blue Sentinel Lymph Node Biopsy in Melanoma”. Oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

Współpraca z autorem przy koncepcji pracy, metodologii, analizie wyników oraz poprawkach merytorycznych publikacji.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek Tomasza Cwalińskiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek Tomasza Cwalińskiego przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.



(podpis współautora)

Gdańsk, dnia 18.02.2022

Dr n. med. Wojciech Połom  
(tytuł zawodowy, imię i nazwisko)

## OŚWIADCZENIE

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.....  
Wojciech Połom

(podpis współautora)

**To: Karol Polom, Clinic of Surgical Oncology  
Smoluchowskiego 17, Gdańsk 80-214, Poland**

Siena, date 18.02.2022

Luigi Marano, MD PhD

**STATEMENT**

As a co-author of the work entitled „Fluorescence Imaging Using Methylene Blue Sentinel Lymph Node Biopsy in Melanoma”. I certify that my own substantive contribution to the preparation, conduct and development of research and presentation of the work in the form of a publication are:

Cooperation with the author on the concept of the work, analysis and substantive corrections of the work.

At the same time, I consent to the submission of the above-mentioned work by Tomasz Cwaliński as part of a doctoral dissertation in the form of a thematically coherent set of articles published in scientific journals.

I declare that the independent and separable part of the above-mentioned work shows the individual contribution of Tomasz Cwaliński in the development of the concept, implementation of the experimental part, and development and interpreting the results of this work.



co author signature

Gdańsk, dnia 18.02.2022

Dr n. med. Maciej Świerblewski  
(tytuł zawodowy, imię i nazwisko)

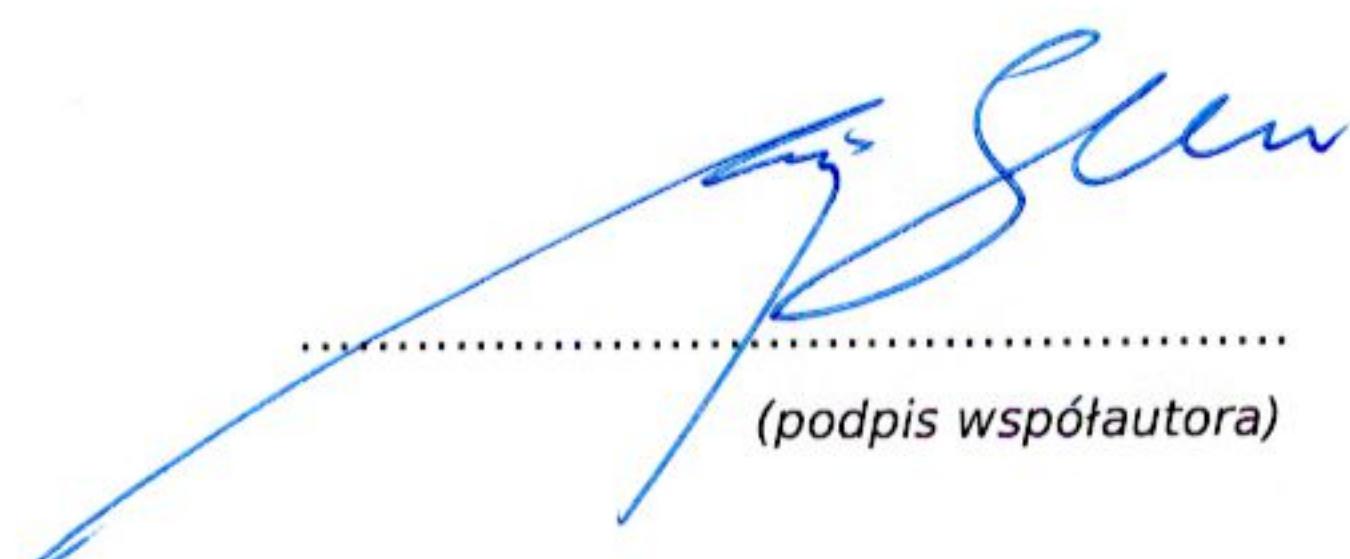
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Współpraca z autorem przy koncepcji pracy oraz analizie wyników.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek Tomasza Cwalińskiego jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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(podpis współautora)

Gdańsk, dnia 18.02.2022

Dr n. med. Kamil Drucis  
(tytuł zawodowy, imię i nazwisko)

## OŚWIADCZENIE

Jako współautor pracy pt. „Fluorescence Imaging Using Methylene Blue Sentinel Lymph Node Biopsy in Melanoma”. Oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

Współpraca z autorem przy analizie wyników oraz poprawkach merytorycznych publikacji.

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dr n. med. Kamil Drucis  
specjalista chirurgii ogólnej  
specjalista chirurgii onkologicznej  
2569839

(podpis współautora)

Florence , date 18.02.2022

Giandomenico Roviello, MD PhD

**STATEMENT**

As a co-author of the work entitled "Methylene Blue — Current Knowledge, Fluorescent Properties, and Its Future Use"; I certify that my own substantive contribution to the preparation, conduct and development of research and presentation of the work in the form of a publication are:

Cooperation with the author on the concept of the work, analysis and substantive corrections of the work.

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I declare that the independent and separable part of the above-mentioned work shows the individual contribution of Tomasz Cwaliński in the development of the concept, implementation of the experimental part, and development and interpreting the results of this work.



co-author signature

Natalia Cwalina MD

**STATEMENT**

As a co-author of the work entitled "Methylene Blue — Current Knowledge, Fluorescent Properties, and Its Future Use"; I certify that my own substantive contribution to the preparation, conduct and development of research and presentation of the work in the form of a publication are:

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*Natalia Cwalina.....*

co-author signature

Gdańsk, dnia 18.02.2022

Prof. dr hab. n. med. Leszek Kalinowski  
(tytuł zawodowy, imię i nazwisko)

## OŚWIADCZENIE

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(podpis współautora)

**To: Karol Polom, Clinic of Surgical Oncology  
Smoluchowskiego 17, Gdańsk 80-214, Poland**

Siena , date 18.02.2022

Franco Roviello MD, FACS

**STATEMENT**

As a co-author of the work entitled "Methylene Blue — Current Knowledge, Fluorescent Properties, and Its Future Use"; I certify that my own substantive contribution to the preparation, conduct and development of research and presentation of the work in the form of a publication are:

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*Fran Roviello*

.....  
co-author signature

)

Gdańsk, dnia 18.02.2022

Dr hab. n. med. Karol Połom  
(tytuł zawodowy, imię i nazwisko)

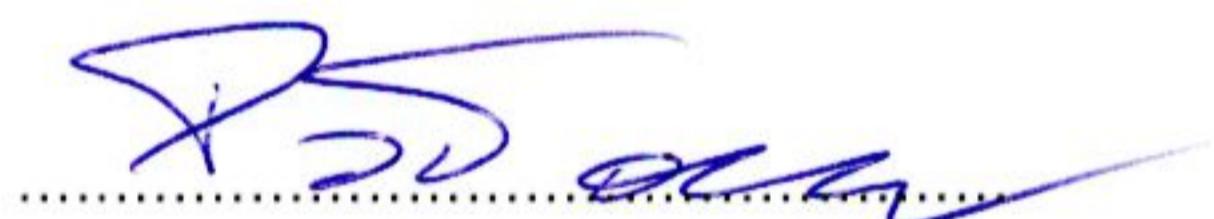
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(podpis współautora)