The handle http://hdl.handle.net/1887/44147 holds various files of this Leiden University dissertation

**Author:** Berg, Nynke van den  
**Title:** Advancing surgical guidance: from (hybrid) molecule to man and beyond  
**Issue Date:** 2016-11-10
CHAPTER 9

MULTISPECTRAL FLUORESCENCE IMAGING DURING ROBOT-ASSISTED LAPAROSCOPIC SENTINEL NODE BIOPSY: A FIRST STEP TOWARDS A FLUORESCENCE-BASED ANATOMIC ROADMAP

ABSTRACT

BACKGROUND  During (robot-assisted) sentinel node (SN) biopsy procedures, intraoperative fluorescence imaging can be used to enhance radioguided SN excision. For this combined pre- and intraoperative SN identification was realized using the hybrid SN tracer, indocyanine green-$^{99m}$Tc-nanocolloid. Combining this dedicated SN tracer with a lymphangiographic tracer such as fluorescein may further enhance the accuracy of SN biopsy.

OBJECTIVE  Clinical evaluation of a multispectral fluorescence guided surgery approach using the dedicated SN tracer ICG-$^{99m}$Tc-nanocolloid, the lymphangiographic tracer fluorescein, and a commercially available fluorescence laparoscope.

DESIGN, SETTING, AND PARTICIPANTS  Pilot study in ten patients with prostate cancer. Following ICG-$^{99m}$Tc-nanocolloid administration and preoperative lymphoscintigraphy and single photon emission combined with computed tomography imaging, the number and location of SNs were determined. Fluorescein was injected intraprostatically immediately after the patient was anesthetized. A multispectral fluorescence laparoscope was used intraoperatively to identify both fluorescent signatures.

SURGICAL PROCEDURE  Multispectral fluorescence imaging during robot-assisted radical prostatectomy with extended pelvic lymph node dissection and SN biopsy.

MEASUREMENTS  (1) Number and location of preoperatively identified SNs. (2) Number and location of SNs intraoperatively identified via ICG-$^{99m}$Tc-nanocolloid imaging. (3) Rate of intraoperative lymphatic duct identification via fluorescein imaging. (4) Tumor status of excised (sentinel) lymph node(s). (5) Postoperative complications and follow-up.

RESULTS AND LIMITATIONS  Near-infrared fluorescence imaging of ICG-$^{99m}$Tc-nanocolloid visualized 85.3% of the SNs. In 8/10 patients, fluorescein imaging allowed bright and accurate identification of lymphatic ducts, although higher background staining and tracer washout were observed. The main limitation is the small patient population.

CONCLUSION  Our findings indicate that a lymphangiographic tracer can provide additional information during SN biopsy based on ICG-$^{99m}$Tc-nanocolloid. The study suggests that multispectral fluorescence image-guided surgery is clinically feasible.
INTRODUCTION

Fluorescence imaging is rapidly finding its way into the operating theatre. A wide spectrum of fluorescent tracers has already been explored in clinical (first-in-human) studies as either free dye or a dye-functionalized targeting agent [1]. In the field of urology, fluorescence guidance has amongst others been used for sentinel node (SN) biopsy of prostate cancer [2, 3]. For this procedure, the near-infrared fluorescent dye indocyanine green (ICG), especially in the form of the hybrid tracer ICG-\textsuperscript{99mTc}-nanocolloid was shown to enhance the more traditional radioguided \textsuperscript{99mTc}-nanocolloid-based SN biopsy procedure [4-6].

During the widely applied radioguided SN biopsy procedure of for example melanoma and breast cancer, intraoperatively blue dye is injected to allow the surgeons to optically define the lymphatic flow (lymphangiography) of a tumor; lymphangiographic tracers such as blue dye help to visualize the lymphatic ducts that run from the injection site to the SN [7, 8]. As alternatives to blue dye, the visible fluorescent dye fluorescein and the near-infrared fluorescent dye ICG in its ‘free’ form have been used [4,9-11]. While the fluorescent alternatives provide enhanced detection sensitivity compared to blue dye, their detection can be complex in combination with ICG-\textsuperscript{99mTc}-nanocolloid. In this context, the concept of multi-color, or multispectral, fluorescence imaging can provide a solution.

Multispectral imaging involves concurrent use of multiple fluorescent dyes to highlight various molecular, physiological, and/or anatomical features [12, 13]. Factors critical in achieving successful multispectral imaging guidance are: 1) the clinical availability of fluorescence tracers that do not spectrally overlap (e.g. fluorescein ($\lambda_{\text{em max}} = 515$ nm) and ICG ($\lambda_{\text{em max}} = 820$ nm)); and 2) a (laparoscopic) fluorescence camera capable of detecting different fluorescence emissions. It should be noted that some of the currently available near-infrared fluorescence laparoscopes, designed for combined use with ICG, have evolved from laparoscopes developed for photodynamic diagnostics, which detects fluorescence emitted in the visual region [14].

In this study we investigated if intraoperative lymphangiography with fluorescein can provide additional guidance during ICG-\textsuperscript{99mTc}-nanocolloid-based SN biopsy for prostate cancer. To this end we required intraoperative multispectral imaging. Hence, the secondary aim of the study was to prove the clinical feasibility of intraoperative multispectral fluorescence imaging.
METHODS

PRECLINICAL EVALUATION OF FLUORESCIN AS LYMPH-ANGIOGRAPHIC AGENT

Initial preclinical experiments using fluorescein are described and discussed in the supporting information.

CLINICAL EVALUATION OF THE MULTISPECTRAL FLUORESCENCE IMAGING APPROACH

PATIENTS

Between October 2013 and August 2015, ten patients with intermediate or high-risk prostate cancer with a >5% risk of lymph node metastases as estimated using the Briganti nomogram [15] were included in a clinical study approved by the local medical ethical committee (Dutch trial register: NTR4451) after they provided written informed consent.

Patients were scheduled for robot-assisted radical prostatectomy (RARP) and SN biopsy followed by an extended pelvic lymph node dissection (ePLND). The patient characteristics are shown in Table 1.

PREOPERATIVE PROCEDURE

A detailed description of ICG-99mTc-nanocolloid preparation, the injection procedure and preoperative imaging approach is provided in the supporting information. In brief, ICG-99mTc-nanocolloid (204.49 MBq, range 191.56-218.20) was injected into the prostate 4.75 h before surgery (range 3.5-5.5) under transrectal ultrasound guidance. Then preoperative SN mapping using a combination of lymphoscintigraphy and single photon emission computed tomography combined with computed tomography (SPECT/CT) was performed to determine the number and location of the SN(s).

SURGICAL PROCEDURE

HARDWARE

Surgical procedures were performed by one surgeon (HvdP) using the da Vinci Si(i) surgical system (Intuitive Surgical Inc., Sunnyvale, CA, US).

Multispectral fluorescence imaging requires multiple dyes that do not overlap spectrally for sequential excitation and detection. We used the Image 1 HUB HD + D-light P system (KARL STORZ Endoskope GmbH & Co. KG, Tuttingen, Germany) with customized filter settings [5] for sequential visualization of ICG and fluorescein. ICG is excited (780 nm) and detected (820 nm) in the near-infrared mode (Supplementary Figure 1A, B), whereas fluorescein is excited (488 nm) and detected (515 nm) in the autofluorescence (AF) mode (Supplementary Figure 1A, C) [1].
Table 1 Patient characteristics, pathology and follow-up

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Pre-operative PSA (ng/mL)</th>
<th>cProstate size (cc)</th>
<th>cTNM</th>
<th>cgProstate-based Gleason sum score</th>
<th>pTNM, pR</th>
<th>Pathology data</th>
<th>Post-operative complications (Clavien-Dindo)</th>
<th>Biochemical recurrence (PSA (ng/mL))</th>
<th>Adjuvant therapy</th>
<th>Follow-up (m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>57</td>
<td>7.4</td>
<td>11</td>
<td>cT3bN0M0</td>
<td>3+4=7</td>
<td>pT3aN0Mx, pR0</td>
<td>3+4=7</td>
<td>4</td>
<td>10</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>73</td>
<td>3.4</td>
<td>19</td>
<td>cT3aN0M0</td>
<td>3+4=7</td>
<td>pT2cN0Mx, pR0</td>
<td>3+4=7</td>
<td>5</td>
<td>13</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>68</td>
<td>12.7</td>
<td>25</td>
<td>cT2cN0M0</td>
<td>3+5=8</td>
<td>pT4aN1M1a, pR0</td>
<td>3+5=8</td>
<td>3</td>
<td>22</td>
<td>3 / 18</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>69</td>
<td>10.8</td>
<td>37</td>
<td>cT2aN0M0</td>
<td>3+4=7</td>
<td>pT2cN0Mx, pR0</td>
<td>3+4=7</td>
<td>1</td>
<td>6</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>5.2</td>
<td>92</td>
<td>cT3aN0M0</td>
<td>4+5=9</td>
<td>pT2cN0Mx, pR0</td>
<td>3+3=6</td>
<td>9</td>
<td>11</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>15.0</td>
<td>37</td>
<td>cT2bN0M0</td>
<td>4+3=7</td>
<td>pT3aN0Mx, pR0</td>
<td>3+5=8</td>
<td>4</td>
<td>11</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>70</td>
<td>9.4</td>
<td>52</td>
<td>cT2aN0M0</td>
<td>4+3=7</td>
<td>pT2cN0Mx, pR0</td>
<td>4+3=7</td>
<td>6</td>
<td>12</td>
<td>0 / 0</td>
<td>-</td>
</tr>
<tr>
<td>8</td>
<td>66</td>
<td>6.3</td>
<td>44</td>
<td>cT1cNxMx</td>
<td>3+4=7</td>
<td>pT2cN1Mx, pR0</td>
<td>3+4=7</td>
<td>1</td>
<td>19</td>
<td>0 / 1</td>
<td>I</td>
</tr>
<tr>
<td>9</td>
<td>66</td>
<td>9.4</td>
<td>40</td>
<td>cT3aN0M0</td>
<td>3+4=7</td>
<td>pT2aN1M0, pR1</td>
<td>3+4=7</td>
<td>7</td>
<td>17</td>
<td>2 / 17</td>
<td>II</td>
</tr>
<tr>
<td>10</td>
<td>65</td>
<td>6.6</td>
<td>70</td>
<td>cT2cN0M0</td>
<td>3+4=7</td>
<td>pT3aN0Mx, pR0</td>
<td>3+4=7</td>
<td>12</td>
<td>11</td>
<td>0 / 0</td>
<td>-</td>
</tr>
</tbody>
</table>

Average 67 8.6 55.6
Total 52 132 5 / 36

PSA = prostate specific antigen; c = clinical; p = pathological; T = tumor; N = node; M = metastasis; # = number; SNs = sentinel nodes; LNs = lymph nodes. *Patient follow-up elsewhere.
The fluorescence laparoscope was inserted via the assistant-port. The TilePro function of the da Vinci S(i) robot was utilized to present the fluorescence imaging data to the urologist.

**INTRAOPERATIVE PROCEDURE**

Clinical grade fluorescein (fluoresceite, 100 mg/mL) was obtained from Alcon Nederland B.V. (Gorinchem, the Netherlands). For injection of fluorescein, a total volume of 2.0 mL (200 mg fluorescein) was extracted from the vial and injected into the prostate similar to as described for ICG-$^{99m}$Tc-nanocolloid. The aim was to place the fluorescein deposits at the same location as for the hybrid tracer. Then the surgical field was sterilized.

Before incision, preoperatively acquired SPECT/CT images and the corresponding three-dimensional volume-rendered image were used as a virtual roadmap. Better knowledge of the location of the SN(s) with regard to the patient’s anatomic context was achieved by scrolling through the images. These images served as the initial starting point for intraoperative SN localization. After entering the area of interest, the SNs (and the lymphatic ducts) were visualized via fluorescence imaging of ICG-$^{99m}$Tc-nanocolloid (near-infrared mode). The afferent lymphatic ducts (and SNs) were visualized via fluorescence imaging of fluorescein in the AF mode. As described previously, after excision of the SN(s), ex vivo gamma tracing was performed to confirm SN removal [5].

Following completion of SN biopsy, ePLND was performed, followed by RARP as described by KleinJan et al. [5]. In patients in whom preoperative SN mapping did not reveal a SN on one side, only an ePLND on that side was performed.

**SCORING OF THE SENTINEL NODE AND LYMPHANGIOGRAPHIC FINDINGS**

Intraoperatively, for each SN it was scored if 1) it could be visualized via ICG-$^{99m}$Tc-nanocolloid (yes/no) or fluorescein (yes/no) imaging; and 2) if the afferent lymphatic duct(s) could via fluorescein (yes/no) or ICG-$^{99m}$Tc-nanocolloid (yes/no) imaging. The general distribution of the two tracers in the surgical field was also evaluated.

**(HISTO-)PATHOLOGICAL EXAMINATION OF THE TUMOR STATUS**

The prostate and the excised nodes were processed and evaluated for the presence and localization of tumor tissue as previously described [5].

**EX VIVO MULTISPECTRAL FLUORESCENCE IMAGING OF THE INJECTION SITE (PROSTATE) AND NODAL SAMPLES**

To confirm the presence of both fluorescent dyes, samples were evaluated ex vivo using the multispectral fluorescence laparoscope.
### Table 2. Intraoperative imaging findings

<table>
<thead>
<tr>
<th>Patient</th>
<th>Injection hybrid tracer - start operation (hours)</th>
<th>Side that was operated on first</th>
<th># SNs NIR fluorescent in vivo (ex vivo) L + R</th>
<th># SNs with a fluorescein positive lymphatic duct L + R</th>
<th>Location excised SNs</th>
<th>Location SNs to which a lymphatic duct was seen</th>
<th>Location SNs not excised</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.5</td>
<td>L</td>
<td>1 (1) + 2 (2)</td>
<td>1 + 0</td>
<td>L: ext iliac R: int iliac</td>
<td>L: presacral</td>
<td>R: -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>5.5</td>
<td>R</td>
<td>2 (2) + 3 (3)</td>
<td>0 + 1</td>
<td>L: obt fossa (2)</td>
<td>R: int iliac</td>
<td>ext iliac, pararectal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>3.5</td>
<td>L</td>
<td>1 (1) + 1 (1)</td>
<td>0 + 0</td>
<td>L: int iliac R: int iliac</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>5.5</td>
<td>L</td>
<td>1 (1) + nonvis</td>
<td>1 + nonvis</td>
<td>L: int iliac R: -</td>
<td>L: int iliac</td>
<td>L: presacral</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>4.5</td>
<td>R</td>
<td>1 (2) + 2 (3)</td>
<td>0 + 3</td>
<td>L: obt fossa, ext iliac R: obt fossa, int iliac, common iliac trunk</td>
<td>L: -</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>L</td>
<td>1 (1) + 3 (3)</td>
<td>1 + 2</td>
<td>L: int iliac R: int iliac</td>
<td>L: obt fossa, ext iliac, common iliac trunk</td>
<td>R: int iliac, ext iliac</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>4.5</td>
<td>L</td>
<td>1 (3) + 0 (1)</td>
<td>1 + 0</td>
<td>L: obt fossa (2)</td>
<td>R: int iliac</td>
<td>L: ext iliac</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>4.5</td>
<td>L</td>
<td>1 (1) + nonvis</td>
<td>0 + nonvis</td>
<td>L: obt fossa R: -</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>4.5</td>
<td>R</td>
<td>1 (1) + 4 (4)</td>
<td>0 + 2</td>
<td>L: common iliac trunk R: obt fossa (2) ext iliac, near umbilical ligamentum</td>
<td>L: - R: obt fossa (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>5.5</td>
<td>L</td>
<td>3 (3) + 1 (1)</td>
<td>3 + 0</td>
<td>L: obt fossa (3) R: obt fossa</td>
<td>L: obt fossa (3)*</td>
<td>R: -</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>4.75</td>
<td></td>
<td>2.9 (3.4)</td>
<td>1.5</td>
<td>3,4</td>
<td>0,5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>4.75</td>
<td></td>
<td>29 (34)</td>
<td>15</td>
<td>34</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

In patients 2 and 10, 1 and 3 SNs were not evaluated for fluorescein-based visualization of the lymphatic ducts. Next to being stained with ICG-99mTc-nanocolloid, this SN was also fluorescein positive; * Next to being stained with fluorescein, these lymphatic ducts were also ICG-99mTc-nanocolloid positive. L = left; R = right; # = number; SN = sentinel node; NIR = near-infraread; nonvis = non-visualization on preoperative imaging; ext = external; int = internal; obt = obturator.
POSTOPERATIVE COMPLICATIONS AND FOLLOW-UP

Postoperative complications (within 90 d after surgery) were scored using the Clavien-Dindo classification [16, 17]. Patients were also evaluated for biochemical recurrence (prostate specific antigen > 0.1 ng/mL) at follow-up.

RESULTS

Following intraprostatic ICG-\(^{99m}\)Tc-nanocolloid administration, preoperative imaging revealed 36 SNs in ten patients (mean 3.6; range 1-5; Figure 1 and Table SI1), of which 85.3% were visualized intraoperatively via real-time (video-rate) near-infrared fluorescence imaging (in 10/10 patients; Table 2, Figure 2). For superficially located SNs, fluorescein visualized lymphatic ducts to 44.1% of the SNs (in 8/10 patients; Table 2, Figure 2). This visualization was also in real-time and was achieved with good contrast and high resolution. In one patient bilateral lymphatic ducts were visualized via fluorescein imaging. In two patients, no lymphatic ducts could be visualized via fluorescein imaging, which may be a result of prostate site mismatch for fluorescein and ICG-\(^{99m}\)Tc-nanocolloid administration (Figure 3).

In one patient the lymphatic ducts could be identified via imaging of both fluorescein and ICG-\(^{99m}\)Tc-nanocolloid (Table 2, Figure 2A, B). In another patient fluorescein was also detected in an SN containing ICG-\(^{99m}\)Tc-nanocolloid (Table 2, Figure 2D), which was confirmed ex vivo in the excised SN specimen (Figure 3).

Figure 1. Preoperative sentinel node mapping. Following ICG-\(^{99m}\)Tc-nanocolloid administration, lymphoscintigrams were acquired at A) 15 min and B) 2 h after injection, followed by single photon emission computed tomography (SPECT) and computed tomography (CT) image acquisition. C) Following reconstruction, SPECT and CT images were fused and a three-dimensional volume rendering was generated. D) Axial SPECT/CT slices show the sentinel nodes being located in the external iliac (left and right) and the internal iliac (right). SN = sentinel node.
Figure 2. Intraoperative multispectral fluorescence imaging. Laparoscopic fluorescence imaging was performed of the areas of interest (left column). A) Visualization of a lymphatic duct via both near-infrared fluorescence imaging of the hybrid tracer (middle column; arrow) and visible fluorescence imaging of fluorescein (right column; arrow); B) The lymphatic duct seen under A ended in a sentinel node visualized via fluorescence imaging of the hybrid tracer (middle column; asterisk). No fluorescein was found in the sentinel node here (right column); C) Example of a sentinel node that could be visualized via fluorescence imaging of the hybrid tracer (right column; asterisk) whereas via fluorescein imaging clear lymphatic ducts were visualized running to this sentinel node (right column; arrows); D) Clear sentinel node visualization via both near-infrared fluorescence imaging of the hybrid tracer (middle column; asterisk) and fluorescence imaging of fluorescein (right column; asterisk). With fluorescein also two clear lymphatic ducts running to the sentinel node were visualized (right column; arrows).
For this particular surgical procedure, manipulation of the prostate (injection site) resulted in release of fluorescein into the surgical field. Moreover, when disconnecting the prostate from the bladder, fluorescein-containing urine started to leak into the surgical field. In addition, a strong overall background signal was encountered with fluorescein, possibly as a result of shunting; in most patients, the injection of a tracer into the prostate results in some loss into the vascular system. It should be mentioned that this background signal was not a consequence of autofluorescence, since these signals were not visible in the AF mode when fluorescein was not present.

Nodal metastases were found in three patients, with a false-negative SN in one man (a metastatic lymph node was found in the ePLND but no metastases in the concomitant SN) (Table 1).

Postoperative complications within 90 days after surgery were seen in two patients, epididymitis and hematoma with a Clavien-Dindo score of II, and I, respectively (Table 1). Biochemical recurrence occurred in two patients (Table 1).

**DISCUSSION**

We present a first-in-human multispectral fluorescence imaging approach in which ICG-$^{99m}$Tc-nanocolloid-based SN identification was supported by additional lymphangiographic guidance provided by fluorescein. We demonstrated that both fluorescent dyes could be detected efficiently using a commercially available (fluorescence) laparoscope.
Unfortunately, the proof-of-concept nature of this study, with a relatively small number of patients included, makes it hard to make statements regarding the clinical impact of this technology.

The small molecule fluorescein was injected directly before the start of the operation and rapidly drained through the lymphatic system without specific accumulation in the (sentinel) lymph node(s), but brightly staining the lymphatic duct(s) running to the SN(s). The number of lymphatic ducts visualized using this technique was higher than the ductal visualization achieved using ICG-\(^{99m}\text{Tc}\)-nanocolloid (Table 2, Figure 2). Similar to blue dye, another small molecule used for lymphangiography, the time-window in which fluorescein can be detected is limited. Because of this limitation, fluorescein-positive lymphatic ducts could only be observed at the site where the urologist started his exploration (seven patients; Table 2). Presumably the signal on contralateral pelvic side had already disappeared by the time the surgical exploration started. It should also be noted that ‘free’ ICG can be considered a lymphangiographic agent with a relatively short effective time-window [4]. Another shortcoming is that these lymphangiographic tracers potentially suffer from spillage as a result of tissue manipulation during the resection process, and can thus contaminate the surgical field [18]. By contrast, the dedicated SN tracer ICG-\(^{99m}\text{Tc}\)-nanocolloid provides specific uptake and remains present in the SN for more than 29 h after administration [19], providing more logistical freedom. Concomitant use of ICG-\(^{99m}\text{Tc}\)-nanocolloid provides a solid basis for fluorescein to add additional value, without suffering much from its shortcomings, similar to the routine clinical use of blue dye in combination with \(^{99m}\text{Tc}\)-nanocolloid in other malignancies [7, 8].

The hybrid nature of ICG-\(^{99m}\text{Tc}\)-nanocolloid facilitated preoperative checking of correct injection of the hybrid tracer into the prostate, which is not possible with fluorescein. Preoperative imaging also demonstrated that ICG-\(^{99m}\text{Tc}\)-nanocolloid is hepatically cleared, as previously reported for ICG [20]. By contrast, fluorescein is cleared renally, so it has been used as an imaging agent for ureter visualization [21]. This renal clearance of fluorescein was not considered a limitation during the SN biopsy procedure, but did lead to contamination of the surgical field during prostatectomy. This could affect the use of future tumor-receptor targeted tracers, as it indicates that fluorescence guidance towards cancer in the prostate (or bladder) may suffer from false-positive signals when a renally cleared tracer is used [18]. Depending on the excitation light and the detection filters used in the laparoscope, such a signal may also limit the identification of surgical resection margins.

Intraoperative fluorescein detection was straightforward, suggesting that use of a near-infrared fluorescence tracer is not a perquisite. Of course, searching for an additional fluorescence signal took additional effort and time, but because the same clinical grade laparoscope could be used, this process was rather intuitive. In comparison to the conventional radioguided SN approach, the additional costs of ICG-\(^{99m}\text{Tc}\)-nanocolloid is negligible [22], and use of fluorescein involves only minor additional costs [11]. As the laparoscope used for fluorescence imaging also provides high-definition white light
imaging, its conversion to multispectral fluorescence imaging can be covered when upgrading the clinical modalities routinely used for laparoscopic surgery. However, one limitation is that a multispectral laparoscope is not yet available as part of the robotic platform.

Although some will be of the opinion that combining a lymphangiographic tracer with a dedicated SN tracer can lead to further refinement of the SN biopsy procedure, others may believe that this will make the procedure more complex. However, bearing in mind the large number of (tumor-receptor) targeted fluorescent tracers with varying fluorescent signatures that have been applied in clinical studies [1], and the rapid development of new imaging tracers, the ability to perform multispectral imaging lays a basis for future surgical guidance applications. Ideally image guidance will facilitate identification not only of diseased areas but also of distinct anatomic landmarks such as ligaments, vessels, nerves, and/or ureters. A potential future application of multispectral fluorescence guidance could be nerve-sparing prostatectomy using tracers targeting prostate-specific membrane antigen [23] and nerves [24]. A read-out of two (or more) features would allow the generation of an intraoperative multispectral fluorescence imaging-based roadmap highlighting different (diseased) anatomic structures in real time. This could help to improve surgical accuracy and reduce procedure-associated morbidity.

CONCLUSION

Simultaneous use of a dedicated SN tracer and a lymphangiographic tracer during robot-assisted laparoscopic SN biopsy for prostate cancer is feasible. Ultimately this multispectral surgical guidance concept will allow the generation of an intraoperative multispectral fluorescence imaging-based roadmap that can highlight different (diseased) anatomical structures in real-time.

SURGERY IN MOTION

The Surgery in Motion video accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.eururo.2016.06.012 and via www.europeanurology.com.

REFERENCES

3. van den Berg NS, van Leeuwen FW, van der Poel HG. Fluorescence guidance in urologic
SUPPORTING INFORMATION

METHODS

HYBRID TRACER PREPARATION, INJECTION AND PREOPERATIVE SENTINEL NODE MAPPING

\(^{99m}\text{Tc}\)-nanocolloid was prepared by adding 2.0 mL pertechnetate (≈300 MBq) to a vial of nanocolloid (GE Healthcare, Eindhoven, the Netherlands). The hybrid tracer was then formed by adding 0.05 mL (0.25 mg) of ICG solution (5.0 mg/mL; Pulsion Medical, Feldkirchen, Germany) to this vial. After formation of ICG-\(^{99m}\text{Tc}\)-nanocolloid, the total volume was subtracted from the vial and diluted with saline to a total volume of 2.0 mL in the syringe. Procedures were performed in accordance with the Dutch guidelines for good manufacturing practice and with approval of the local pharmacist.

On average 4.75 h before surgery (range 3.5-5.5) ICG-\(^{99m}\text{Tc}\)-nanocolloid (average of 204.49 MBq (range 191.56-218.20)) was injected under transrectal ultrasound guidance into the prostate. Randomization was performed between an intratumoral injection (2 deposits of 1.0 mL on site where the dominant lesion was located) and an intraprostatic injection (4 deposits of 0.5 mL; 2 on the left site of the gland and 2 on the right site).

The injection was followed by preoperative SN mapping in the form of lymphoscintigraphy (15 min and 2 h post-hybrid-tracer-injection; 5 min acquisition time per image) and single photon emission computed tomography combined with computed tomography (SPECT/CT; approximately 2.5 h post-hybrid-tracer-injection, 25 min acquisition time)) as described previously [1]. After fusion of the SPECT and CT images, a 3D SPECT/CT-based volume rendering reconstruction was created using Osirix medical imaging software (Pixmeo, Geneva, Switzerland). Images were analyzed by an experienced nuclear medicine physician and for each patient the number and location of visualized SNs was determined (Table SI1).

ABSORPTION AND EMISSION SPECTRA MEASUREMENTS

Absorption and emission spectra of ICG-human serum albumin (concentration: 13.38 \(\mu\)M) and fluorescein-human serum albumin (concentration: 1.12 \(\mu\)M) were measured using an Ultrospec 3000 UV/Vis spectrophotometer (Pharmacia Biotech/GE Healthcare Europe GmbH, Eindhoven, The Netherlands) and an LS55 fluorescence spectrometer (PerkinElmer, Groningen, The Netherlands). Solutions were prepared in a 3 mL quartz cuvet (Hellma GmbH & Co. KG, Müllheim, Germany).

PRECLINICAL STUDY

Non-tumor bearing male TRAMP mice (n=3) were injected with 10-20 \(\mu\)L of fluorescein solution (1 mg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) into the left lobe of the prostate of the mouse as previously described [2]. After dye administration, the injection site was massaged for up to 1 min.

For intraoperative detection of fluorescein dynamic fluorescence imaging was performed using a self-made fluorescence camera exciting fluorescein (excitation 488 nm; emission >520 nm). A total of six inguinal lymph node(s) were evaluated.
RESULTS

ABSORPTION AND EMISSION SPECTRA MEASUREMENTS

The absorption and emission spectra of ICG and fluorescein are given in Figure SI1.

Figure SI1. Excitation and emission spectra fluorescein and ICG-human serum albumin. A) Absorption (dashed lines) and emission spectrum of ICG (black) and fluorescein (grey); B) Blue light is used to excite fluorescein; C) Near-infrared light is used to excite ICG.

Figure SI2. Lymphangiography using the visible fluorescence dye fluorescein. Upon fluorescein injection into the mouse prostate (*) real-time lymphatic mapping was found feasible. Within 10 s (A) drainage occurs into the lymphatic vessels (red arrow). Already at 30 s (B) fluorescein reached the lymph node (yellow arrow), which is fully filled roughly 1 min post-injection of fluorescein (C).
PRECLINICAL STUDY

Fluorescence imaging enabled real-time (at video-rate) intraoperative visualization of the lymphatic ducts and the inguinal lymph nodes (Figure SI2). In all mice, drainage from the injection site into the lymphatic ducts could be visualized within 10 s after injection of fluorescein. Staining of the lymph node(s) occurred immediately thereafter. Washout of fluorescein from the nodes was seen >2 min post-injection and suggested a quick migration of the dye through the lymphatic system comparable to what we previously showed for ICG [2].

Table SI1. Preoperative imaging findings

<table>
<thead>
<tr>
<th>Patient</th>
<th>Injected dose (MBq)</th>
<th># SNs early LSG L + R</th>
<th># SNs late LSG L + R</th>
<th># SNs SPECT/CT L + R</th>
<th>Location SNs</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>224.31</td>
<td>1 + 0</td>
<td>2 + 1</td>
<td>2 + 2</td>
<td>L: ext iliac, presacral R: ext iliac, int iliac</td>
</tr>
<tr>
<td>2</td>
<td>202.08</td>
<td>1 + 2</td>
<td>1 + 2</td>
<td>1 + 3</td>
<td>L: obt fossa R: obt fossa, int iliac, ext iliac</td>
</tr>
<tr>
<td>3</td>
<td>208.91</td>
<td>0 + 0</td>
<td>0 + 0</td>
<td>1 + 1</td>
<td>L: obt fossa R: obt fossa</td>
</tr>
<tr>
<td>4</td>
<td>192.29</td>
<td>1 + 0</td>
<td>1 + 0</td>
<td>2 + 0</td>
<td>L: int iliac, presacral R: obt fossa</td>
</tr>
<tr>
<td>5</td>
<td>218.20</td>
<td>1 + 3</td>
<td>1 + 3</td>
<td>1 + 3</td>
<td>L: obt fossa R: obt fossa (2), paracaval</td>
</tr>
<tr>
<td>6</td>
<td>197.07</td>
<td>0 + 0</td>
<td>1 + 2</td>
<td>3 + 2</td>
<td>L: int iliac, presacral (2) R: obt fossa (2)</td>
</tr>
<tr>
<td>7</td>
<td>193.98</td>
<td>2 + 1</td>
<td>2 + 1</td>
<td>3 + 2</td>
<td>L: obt fossa, int iliac, ext iliac R: obt fossa, bifurcation common iliac trunk</td>
</tr>
<tr>
<td>8</td>
<td>200.19</td>
<td>0 + 0</td>
<td>0 + 0</td>
<td>1 + 0</td>
<td>L: obt fossa R: obt fossa</td>
</tr>
<tr>
<td>9</td>
<td>216.35</td>
<td>1 + 3</td>
<td>1 + 3</td>
<td>1 + 3</td>
<td>L: common iliac trunk R: obt fossa, ext iliac, bifurcation common iliac trunk</td>
</tr>
<tr>
<td>10</td>
<td>191.56</td>
<td>2 + 0</td>
<td>2 + 2</td>
<td>2 + 3</td>
<td>L: int iliac, paravesical R: obt fossa, bifurcation common iliac trunk (2)</td>
</tr>
<tr>
<td>Average</td>
<td>204.49</td>
<td>1.8</td>
<td>2.3</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>18</td>
<td>23</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

MBq = Mega Bequerel; LSG = lymphoscintigraphy; SPECT/CT = single photon emission computed tomography combined with computed tomography; L = left; R = right; SN = sentinel node; obt = obturator; int = internal; ext = external.
REFERENCES

