

## Vitamin B2 as a Tracer for Intraoperative Pulmonary Sentinel Node Navigation Surgery

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**Abstract.** *Background: We investigated whether fluorescent agents, especially vitamin B2, can act as tracers for intraoperative pulmonary sentinel node mapping. Materials and Methods: Vitamin B2, fluorescent beads, and green fluorescent protein (GFP) were each injected into pulmonary parenchyma in 4 pigs (experiment 1). The safety of each tracer was also verified in 12 rats (experiment 2). Results: Experiment 1: In all groups, the sentinel lymph node was identified in 3 out of the 4 pigs (75%). Speed of agent dispersion: vitamin B2>GFP>fluorescent beads. Level of fluorescence judged as: vitamin B2>GFP=fluorescent beads. Experiment 2: In all groups, all rats survived until sacrifice without complications. In the fluorescent beads group, the fluorescent beads remained in the blood vessels. Conclusion: Vitamin B2 is inexpensive, safe and easy to apply. It is anticipated that clinical application of vitamin B2 for intraoperative pulmonary sentinel node mapping will become possible.*

The standard surgical therapy for lung cancer is lobectomy and systematic lymph node dissection. However, in 80% of clinical stage IA patients, mediastinal lymph node metastases are not seen on postoperative pathological examination (1). Lymph node dissection is highly useful for an accurate diagnosis of the pathological stage, but in most cases it is not a curative treatment and in some cases, it can even be harmful for patients. The possibility of damage to nerves or vessels during lymph node dissection increases with the extent of the dissection, and the frequency of pulmonary or cardiovascular complications increases. Therefore, it is important to determine which lymph nodes do not need to be dissected.

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The rationale of the sentinel lymph node (SLN) concept is that the lymphatic channels drain into SLNs, which are the first lymph nodes in a chain of nodes to receive the afferent lymphatics of the tumor bed. Therefore, SLNs are an expected site for cancer cell migration. SLN identification is an important procedure in patients with not only melanoma (2) and breast cancer (3) but also lung cancer (4-10). There are several studies about sentinel node navigation surgery (SNNS) in non-small cell lung cancer (NSCLC). The major two methods in current SNNS are dye methods using isosulfan or patent blue (4) *etc.* and radioisotope (RI) method using technetium-99m ( $^{99m}\text{Tc}$ ) colloid (5-7) *etc.* The former can be performed easily and promptly during operation. However, the identification rate is low, especially in lymph nodes with anthracotic pigmentation (4). The latter achieves a high identification rate (5, 6), however, management of radionuclide is difficult in Japan due to legal limitations. In addition, the RI method needs preoperative injection into the lung and there are some complications associated with the injection such as pneumothorax and bleeding.

Recently, some studies have demonstrated the usefulness of fluorescent agents such as indocyanine green (ICG) (8), quantum dots (QDs) (9) and fluorescent beads (10) as novel lymph tracers. However, it is unclear whether such fluorescent agents are safe and effective to identify SLNs in the human body or not. In the present study, animal experiments were performed using three fluorescent agents (vitamin B2, fluorescent beads and green fluorescent protein (GFP)) to investigate the feasibility for clinical application of fluorescent agents as tracers to identify SLNs in lung cancer. We also examined which of the three agents would likely be the best tracer.

### Materials and Methods

**Fluorescent agents.** Vitamin B2, fluorescent beads, and GFP are fluorescent tracers. Vitamin B2 is a biologically active agent that is classified as a water-soluble vitamin. Excitation and emission peaks of vitamin B2 are 444 nm and 535 nm. Absorbed vitamin B2 is transformed into flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) in the human body through the action of cellular

enzymes, with the majority becoming FAD. In this study, therefore, FAD sodium (Flavitan, Toa Eiyo Ltd., Fukushima, Japan) was used. Fluorescent beads used (Fluoresbrite™ carboxylate NYO, 0.05 and 0.2  $\mu$ m; Polysciences, Inc, Warrington, PA, USA) consist of polystyrene latex microspheres, and various sizes can be made to match the intended use. They are reported to be useful for detecting SLNs in animal experiments (10). Excitation and emission peaks of these fluorescent beads are 530 nm and 590 nm. GFP (BD Biosciences Clontech, Palo Alto, CA, USA) is a recombinant protein in which photoprotein isolated from the jellyfish *Aequorea victoria* is prepared with *Escherichia coli*. GFP responds to blue light (400-460 nm) by emitting green light (509 nm).

**Experimental animals.** All animals received humane care in accordance with the "Guidelines for the Care and Use of Laboratory Animals" of Kanazawa University and the "Guide for the Care and Use of Laboratory Animals," prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press, revised in 1996.

**Experiment 1:** Twelve female LWD pigs (Sankyo Labo Service Co. Inc., Toyama, Japan) weighing 30 to 40 kg (mean: 33.5 $\pm$ 3.2 kg) were used. Pigs were given intramuscular injections of ketamine hydrochloride (20 mg/kg; Ketalar, Daiichi Sankyo Propharma, Tokyo, Japan) and underwent endotracheal intubation. Anesthesia was maintained with inhalation administration of halothane (0.5%-1.5%; Fluothane, Takeda Pharmaceutical Company Ltd., Osaka, Japan), and intravenous administration of pancuronium bromide (0.1 mg/kg; Musculax, Schering-Plough K.K., Osaka, Japan) was given as a muscle relaxant for inactivation. With the animal lying on its right side, the chest was opened with a lateral incision made at the left fifth intercostal space. Pigs were randomly assigned to vitamin B2, fluorescent beads (0.05 and 0.2  $\mu$ m), or GFP group (n=4 in each group). Assuming peripheral lung cancer, each of the agents was injected into the subpleural lung parenchyma of the left cranial lobe (11). Subpleural tracer injection was used because the method reportedly improves detection of mediastinal SLNs in NSCLC (12).

The accumulation of each agent between the lymph ducts and lymph nodes was confirmed using an autofluorescence/fluorescence system with a rigid scope of 5.5 mm in diameter (D-Light AF system, KARL STORZ, Tuttlingen, Germany). The system is capable of white light illumination and excitation of aminolevulinic acid (ALA)-induced fluorescence. The system is based on a 300-W xenon lamp with special optics to focus high intensities of light into a liquid light guide, which is optimized for blue light transmission. Observations are made while switching easily between white light mode and ALA mode by a footswitch or a switch mounted on the CCD camera. In ALA mode, wavelengths of 470-800 nm are observed on a monitor with excitation light centered on 380-440 nm (13).

The items evaluated were: (i) identification of lymph nodes that had absorbed fluorescent agent; (ii) time until the agent reached the SLN; and (iii) level of fluorescence (judged macroscopically on the monitor). The dose of each agent was: 0.2 ml ( $\times$ 10 dilution) for vitamin B2, 0.2 ml each for fluorescent beads (0.05 and 0.2  $\mu$ m), and 0.2 ml ( $\times$ 10 dilution) for GFP. When the fluorescence of the mediastinal lymph nodes could not be confirmed, observation of the fluorescence was performed by incising the mediastinal pleura.

**Experiment 2:** The safety and adverse effects of the administration of each fluorescent agent was verified in rats. Twelve male Wister

rats (10 weeks old, mean body weight 256 $\pm$ 20 g, Sankyo Labo Service Co. Inc., Toyama, Japan) were randomly assigned to the Vitamin B2, fluorescent beads, and GFP groups (n=4 in each group). All rats were housed in a temperature-controlled (21°C) environment under a 12-hour light/dark cycle with free access to food and water. Each rat was anesthetized by inhalation of diethyl-ether in an enclosed glass bottle and the anesthesia was maintained with intraperitoneal administration of pentobarbital (60 mg/kg; Nembutal, Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan). In each group, 0.5 ml, at the same concentration as in experiment 1, of vitamin B2, fluorescent beads, or GFP were injected through the tail vein of the rat. The rats were carefully observed for unusual behavior and two each in all groups were sacrificed at weeks 1 and 4 after the injection for evaluation of pathological changes in the liver, kidney and lung. For pathological examination, the dissected organs were fixed in formalin, embedded in paraffin, cut into 4- $\mu$ m sections, and mounted onto glass slides for microscopic examination under hematoxylin and eosin staining.

**Statistics.** Results are expressed as the mean $\pm$ standard deviation. Equality of means was analyzed with the unpaired *t*-test. *P*-values of less than 0.05 were considered significant. All analyses were performed with PASW Statistics 18 software (SPSS Inc., Chicago, USA).

## Results

**Experiment 1.** In ALA mode, vitamin B2 emits yellow-green fluorescence (Figure 1), fluorescent beads emit yellow orange fluorescence (Figure 2a, b), and GFP emits green fluorescence (Figure 3).

Identification of a distinct bright lymph duct, dynamic flow and the draining lymph node was possible in 3 out of the 4 pigs in all 3 groups. A comparison of the time taken for each tracer to reach the lymph nodes is shown in Table I. The time to reach bronchial lymphatics and the first mediastinal lymph nodes that were SLNs was about the same for vitamin B2, 0.05  $\mu$ m fluorescent beads and GFP, but significantly longer for 0.2  $\mu$ m fluorescent beads (*p*<0.0008). The speed of the fluorescent agent dispersion was greater in the following order: vitamin B2>GFP>fluorescent beads. The level of fluorescence was judged macroscopically on the monitor, as vitamin B2>GFP=fluorescent beads. In all cases, the fluorescence of the mediastinal lymph nodes could not be confirmed without pleural incision.

As shown in Figure 1, the fluorescent agents accumulated mainly in the lymph nodes right below the hemiazygos vein when it was injected into the apex of the cranial lobe (Figure 1b), and in the lymph nodes below the hemiazygos vein when it was injected into the cranial lobe near the caudal lobe (Figure 1c). All agents remained in the SLNs over 30 minutes.

As shown in Figure 4, when patent blue, which is normally used in the identification of SLNs (4), was mixed with vitamin B2 and injected directly below the pulmonary pleura, and observed in white light and ALA modes, the patent blue and vitamin B2 were found to flow into the same lymphatic duct.

Table I. Mean time taken for tracers to reach sentinel lymph nodes.

Tracer	Time (seconds)	
	Bronchial lymphatics	First mediastinal LN
Fluorescent beads 0.2 $\mu\text{m}$	557 $\pm$ 60*	683 $\pm$ 59**
0.05 $\mu\text{m}$	151 $\pm$ 43	261 $\pm$ 54
GFP	136 $\pm$ 23	259 $\pm$ 41
Vitamin B2	117 $\pm$ 21	220 $\pm$ 25

LN: Lymph node; GFP: green fluorescent protein. Fluorescent beads of 0.2  $\mu\text{m}$  required significantly longer time to reach each point than other tracers: \* $p$ <0.0006, \*\* $p$ <0.0008.

**Experiment 2.** After injection of the agents, all animals in each group survived until they were sacrificed. No symptoms of shock or other abnormalities were seen shortly after the injection. No abnormal behavior or activity was observed during the observation period in any of the 12 rats. No fluorescent beads were found using the fluorescent camera in the excreted urine at any time. Microscopic examination of the liver, kidney and lung showed no particular changes such as fibrosis, emboli, necrosis, or edema. In the rats that received the fluorescent beads, the beads clearly remained in the blood vessels of all body organs after one week (Figure 2c), but at week 4 this could not be confirmed. In the other two groups, there were no abnormal pathological findings.

## Discussion

SLN mapping in patients with NSCLC was first advocated by Little *et al*. They used isosulfan blue dye as a tracer and obtained identification of SLNs in 47% of the patients (4). This low identification rate was attributed to the difficulty in distinguishing blue-colored lymphatics from adjacent anthracotic tissues. As a consequence, the technique of SLN mapping was refined by the use of other tracers such as magnetite (12, 14) and  $^{99\text{m}}\text{Tc}$ -colloids (4-6).

Liptay *et al*. (5) reported radiolabeled tracer methods that injected  $^{99\text{m}}\text{Tc}$  sulfur colloid into the peritumoral region intraoperatively. Their results were satisfactory and showed an 82% identification rate and a 95% accuracy rate. The procedure described by Nomori *et al*. (6) in which  $^{99\text{m}}\text{Tc}$  tin colloid was injected into the peritumoral region the day before surgery identified SLNs in 87% of the NSCLC patients. False-negative results were observed in 5% of patients by Liptay *et al*. and none in the study of Nomori *et al*. Therefore they argued that the identification of SLNs with  $^{99\text{m}}\text{Tc}$ -colloid is a reliable method of establishing the first site of nodal metastasis in NSCLC.

However, the Cancer and Leukemia Group B 140203 trial, a multi-institutional study, failed to demonstrate the feasibility of an intraoperative SLN mapping technique with a radioactive  $^{99\text{m}}\text{Tc}$  tracer (7). In the study, SLNs were identified in 61.5% of the patients and were found to be accurate in 83.3% of the patients. After all, this SLN mapping procedure was found to be accurate in only 51.2% of the patients. As the reasons for the unexpected low identification rate, they noted technical failure, aerosolization of radioactivity and shine-through effect. SLNs were also poorly identified in patients with a low ratio of forced expiratory volume in 1 second to forced vital capacity because of such conditions as chronic obstructive pulmonary disease (6). In addition, the technique using  $^{99\text{m}}\text{Tc}$ -colloid poses disadvantages from radioactivity for both patients and surgeons.

Recently some studies have demonstrated the usefulness of fluorescent agents in SNNS for lung cancer. Because ICG is known to absorb infrared rays, *in vivo* binding with serum protein, Yamashita *et al*. (8) developed and applied an ICG fluorescence imaging system to NSCLC surgery. SLNs were identified in 80.7% of patients and the overall accuracy rate was 80.7%. Thus, they suggested that video-assisted thoracoscopic ICG fluorescence image-guided surgery is feasible for SLN biopsy and may be a powerful tool to eliminate unnecessary lymph node dissection in patients with lung cancer. On the other hand, some animal experiments with a highly sensitive technique for pulmonary SLN mapping using near-infrared (NIR) fluorescent QDs have been reported (9). Although this technique permits precise real-time imaging even in anthracotic lymph nodes, a limitation regarding the clinical use of NIR QDs for SLN mapping is their potential toxicity. These biological probes contain heavy metals at their cores, with an amphiphilic organic coating. Cadmium, telluride, selenide, and alkyl phosphines exhibit known acute and chronic toxic profiles as isolated heavy metals.

An ideal tracer for SNNS should attain high identification and accuracy rate. Subsequently, it should be safe and inexpensive, and repeatedly useable in an operation. An ideal SNNS procedure should be easy, less invasive and should identify SLNs in a short time. In SNNS for lung cancer, it is better if it can be applicable to video-assisted thoracic surgery. The results of our study suggest that all fluorescent tracers we used are useful for intraoperative pulmonary SLN mapping. When the fluorescent agents were injected into different parenchymas of the lung, it was shown that they drained into different lymph nodes. The time for the agents to reach the SLNs can be controlled by using differently sized agents.

Fluorescent beads have an advantage because they are available in multiple sizes, offering various observation time options. However, fluorescent beads may also have potential toxicity. In the present study, fluorescent beads were clearly



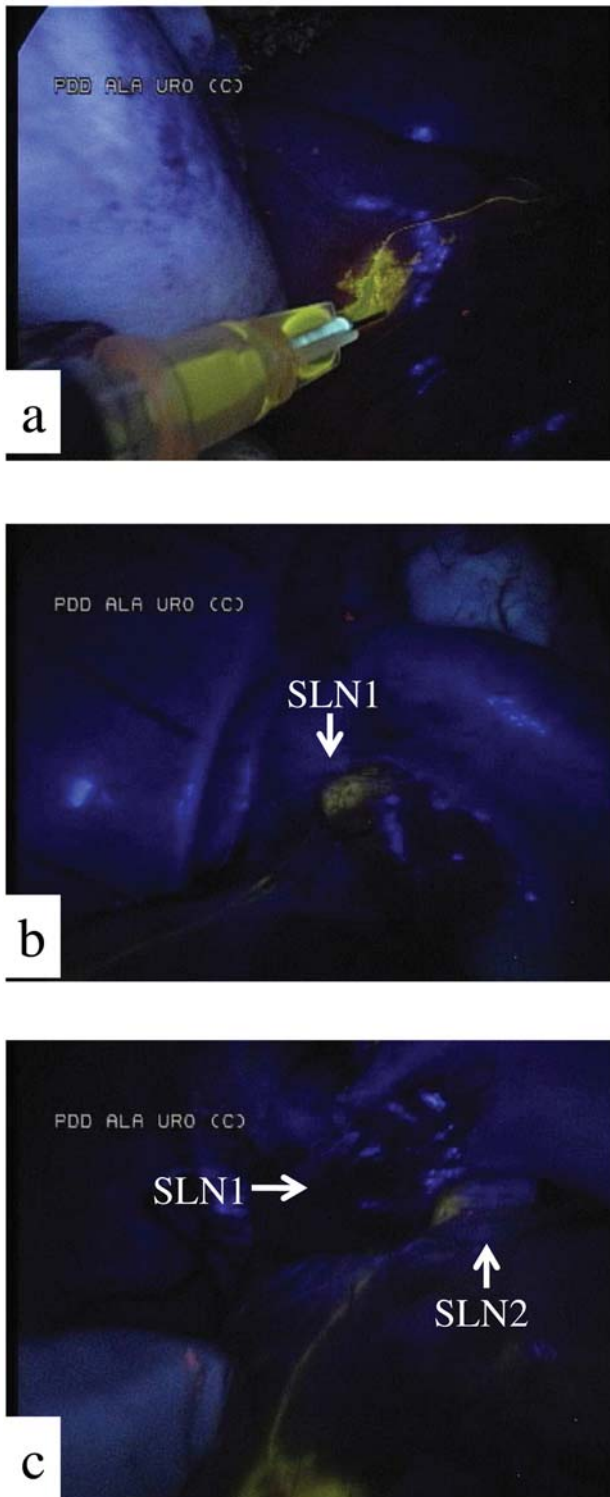


Figure 1. a: Vitamin B2 emits yellow-green fluorescence. b: The fluorescent agents accumulated mainly in the lymph nodes right below the hemiazygos vein when it was injected into the apex of the cranial lobe. c: The fluorescent agents accumulated mainly in the lymph nodes below the hemiazygos vein when it was injected into the cranial lobe near the caudal lobe.

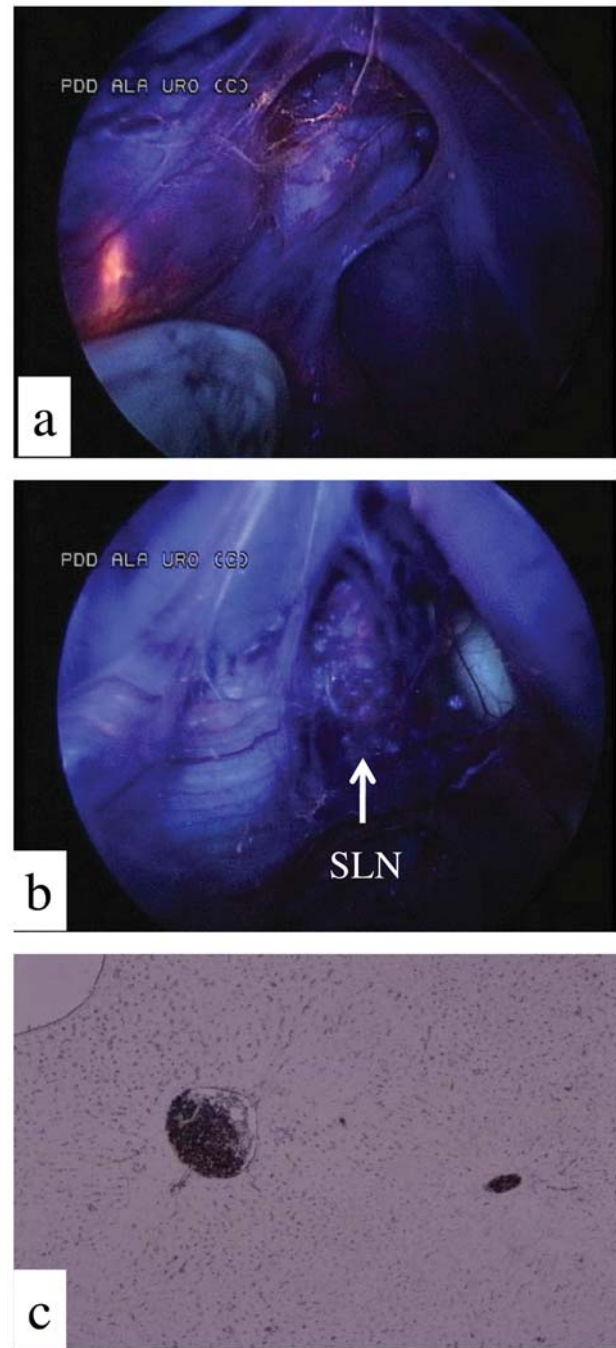


Figure 2. a: Fluorescent beads emit yellow-orange fluorescence. b: Sentinel lymph node (SLN). c: In the rats that received the fluorescent beads, it was found that the beads remained in the blood vessels of all body organs after 1 week following the administration.

evident in the body after 1 week following administration. It is possible that the agents still existed in the body even though there was no clear evidence that they remained at week 4. It is unclear whether the fluorescent beads were evenly dispersed in the entire body or if they were resolved

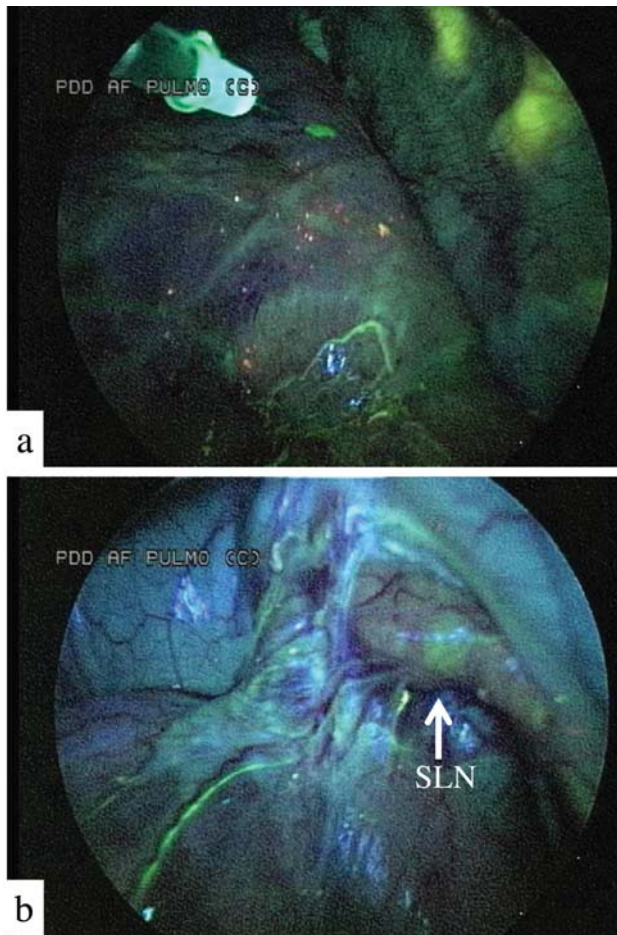


Figure 3. *a*: Green fluorescent protein emits green fluorescence. *b*: Sentinel lymph node (SLN) emits green fluorescence.

in one particular area of the body. Long-term adverse effects still must be further investigated although Ueno *et al.* (10) reported that fluorescent beads do not harm the body. In addition, a single bottle is expensive (about 500 US dollars).

With GFP, allergies are possible, since it is a protein, and since it is an *E. coli* recombinant, its safety in the human body is not assured. Moreover, it is also expensive (about 900 US dollars per bottle).

Among the three agents in our study, vitamin B2 is most highly fluorescent macroscopically on the monitor. Although vitamin B2 is a water-soluble vitamin, it flows into the same lymphatic duct as patent blue, which is normally used in the identification of SLNs. Vitamin B2 is necessary for metabolism of fat, carbohydrate, and protein in the body, and for respiration, formation of red blood cells, production of antibodies, and normal growth. It is maintained at a constant level in major organs, and any excess is eliminated in the urine; thus, it will not cause excessive damage. In addition,

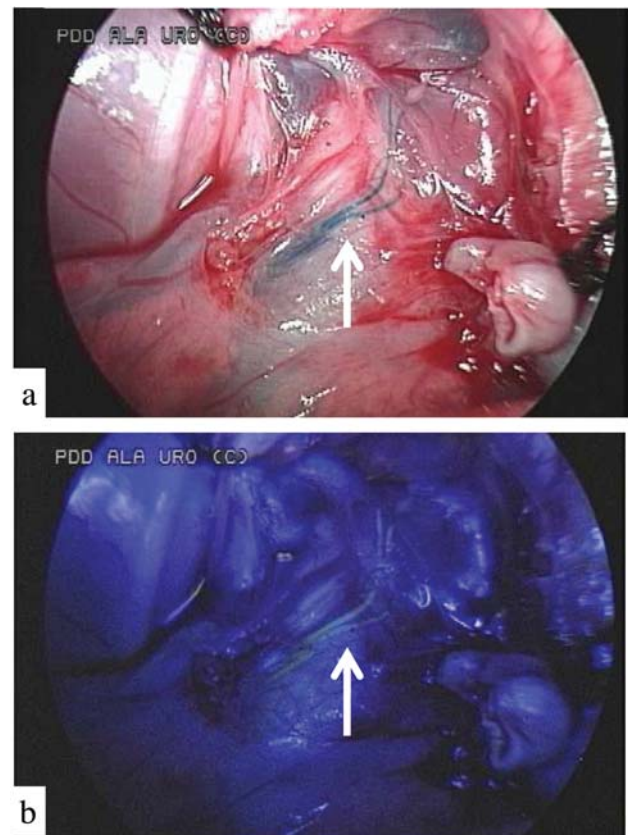


Figure 4. The patent blue and vitamin B2 were found to flow into the same lymphatic duct (arrow).

vitamin B2 is inexpensive (about 10 US dollars per bottle), and of the three agents, it is considered to be the most useful in identifying SLNs.

With regard to limitations, the fluorescence in mediastinal lymph nodes cannot be confirmed without a pleural incision. Even if the SLNs can be identified, no method has yet been established to eliminate dissections based on SLNs. This is because there are limitations to the ability to make a definitive diagnosis of micrometastases on intraoperative pathological assessments, as well as the occurrence of a fair number of overlooked metastases in lung cancer (15). In addition, we injected vitamin B2 subpleurally in two lung cancer patients after obtaining their consent, but the fluorescence was very weak and indistinct. FAD had the weakest fluorescence among vitamin B2 components (riboflavin, FMN, FAD) (16). Future tasks include strengthening the fluorescence, such as by using riboflavin, which has strong fluorescence, and increasing the precision of fluorescence-sensing cameras. It is also possible that the fluorescence intensity is weaker in humans than in pigs because of changes in pH or temperature (16).

In conclusion, the results of our study suggested that fluorescent tracers were useful for intraoperative pulmonary SLN mapping. Among the three tracers studied, vitamin B2 is the most promising agent as it is inexpensive, safe and easy to apply. Therefore, with further studies, it is anticipated that clinical application of vitamin B2 for intraoperative SNNS in NSCLC will become possible.

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