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Intraoperative micrometastases detection using paired-agent fluorescent imaging: trial-and-error protocol development

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ABSTRACT

The status of lymph node is considered a critical prognostic factor for staging and guiding the future adjuvant treatment in many cancer types. The estimation of undetected micrometastases (0.2-2mm diameter) by conventional pathology was around 30-60% cases which has created a demand for the development of more fast and accurate approaches. In response, a paired-agent imaging approach is presented by employing a control imaging agent to allow rapid, quantitative mapping of microscopic cancer cells in lymph nodes to guide pathology sectioning. To identify the most feasible and effective protocol using this approach to detect micrometastases intraoperatively, swine cervical lymph nodes were used to evaluate the potential of different protocols for the agents to diffuse into and out of intact nodes. Aby-029, an anti-EGFR affibody molecule labeled with IRDye-800CW was used as targeted imaging agent, and the IRDye-700DX carboxylate was used as control agent. The time-course paired-agent fluorescence of whole lymph node were recorded to monitor the uptake and washout kinetics. Subsequently, lymph nodes were frozen-sectioned and imaged under an 85-um resolution fluorescence imaging system (Pearl, LICOR) to confirm equivalence of spatial distribution of both agents in the entire node. After much trial-and error, the intranodal infusion staining and rinsing protocol demonstrated promising results that both imaging agents shown strong correlation with each other in the absence of cancer cells ($r=0.99$, $p<0.001$). This methodology indicated the potential of using paired-agent imaging approach to allow rapid and sufficient detection of micrometastases in excised lymph nodes intraoperatively.

Keywords: paired-agent fluorescent imaging, micrometastses, lymph node

1. INTRODUCTION

The lymph node status is one of the most impotant prognostic factor for staging and guiding adjuvant treatment of many cancer types, including breast, melanoma, head and neck and lung cancer[1], as the lymphatic system served as the primary path for tumor cell metastasis[2]. The current standard care of practice is lymph node dissection and followed by pathology examination to evaluate lymph node tumor burden[3]. However, the conventional pathology approach only exanimated less than 1% of lymph node volume, as the lymph node was sectioned as 5-μm-thick slices at 2-mm intervals. Hematoxylin and Eosin (H&E) was then performed on the representative section to provide morphological information for pathologists to identify abnormal cells. This method was aimed to detect tumor cells deposits greater than 2 mm in diameter, defined as macrometastases[4-6], however, there is growing evidence that patients with micrometastases (tumor clusters less than 2 mm in diameter) would benefit from more aggressive therapy. It is estimated that the undetected micrometastases using conventional method was range from 30-60%[7], of which the probability increase with decreasing size of tumor.

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Many studies have shown the increased ability to detect micrometastases in lymph node by taking extensive serial sectioning and immunohistochemistry to enable earlier intervention for guiding therapeutic decision-making [8-10], yet these approaches are not cost-effective and too labor-intensive to be practical. While the importance of micrometastases in prognostic implication remains controversial, the whole procedure of conventional pathology process including fixing, embedding, sectioning and staining still required long process times (>24h), and faster methods suffer from greater false-positive rates[4, 7]. Therefore, a more accurate intraoperative diagnostic approach of aggressive disease without requiring redundant time and resources is needed.

In response, we developed a paired-agent imaging approach by employing a control imaging agent to allow rapid, quantitative mapping of microscopic populations of tumor cells in lymph nodes to provide intraoperative feedback to surgeons with lower false-negative rate. By submerging the excised tissue in paired-agent solution with healthy rat and human lymph nodes, our previous study demonstrated the promising results that the spatial distribution of two imaging agents shown strong correlation with each other in healthy lymph nodes[11]. However, this traditional staining strategy required long process time to reach full permeation of entire lymph nodes in order to acquiring images using fluorescence optical tomography system[12]. This proceeding was to demonstrate the trial-and-error protocol development for staining *ex vivo* lymph node for further investigation of the potential using paired-agent fluorescence imaging to detect micrometastases in excised lymph nodes intraoperatively.

2. METHODS AND RESULTS

2.1 Bisected submerge staining/rinsing protocol evaluation

To evaluate the feasibility of the paired-agent imaging approach, the spatial distribution and time-evolution of the targeted and paired-control imaging agents need to be equivalent throughout staining and rinsing in the absence of cancer cells. In order to shortening the diffusion time of imaging agents into the entire lymph node, lymph nodes were bisected while tissue still remain attached and a tissue pre-staining image was acquired to evaluate background levels caused by autofluorescence. Aby-029, a clinically relevant anti-EGFR affibody molecule labeled with IRDye-800CW (LICOR Biosciences) as targeted imaging agent, and the IRDye-700DX carboxylate (LICOR Biosciences) was used as control agent. A mixture of targeted and control imaging agent for each node was prepared at concentration of 0.2 μM Aby-029 and 0.2 μM IRDye-700DX carboxylate. Lymph nodes were submerging in the paired-agent staining solution that covered with an aluminum foil to protect it from light at room temperature. After 15 min of staining, the lymph node was removed from staining solution then quick dipped into phosphate-buffered saline (PBS) to remove excessive staining solution on the tissue surface. Whole lymph node tissue was then imaged under an 85-μm resolution fluorescence imaging system (Pearl Imager, LICOR Biosciences). Fluorescence at 700-740 nm and 800-840 nm (from 685 and 785 nm excitation, respectively) were acquired to evaluate similarity of diffusion of both targeted and control imaging agents after the staining process. After acquiring images, Lymph nodes were placed back into the mixture staining solution and a total of 4-staining time points were recorded at 15-min interval up to 1 h. Followed by rinsing in PBS and recorded every 15 min for 1 h to monitor the washout of the paired imaging agents. Autofluorescence was removed by subtracting the pre-staining image of respective imaging channels from all subsequent post-staining images. The fluorescence intensity of the targeted and control imaging agents were compared to evaluate uptake and washout during the entire staining/rinsing process. Subsequently, lymph nodes were flash frozen and serial sectioned on a cryostat microtome at 200-μm intervals, the cross-sectional lymph node image of the uptake/retention of control and targeted imaging agent were acquired on the Pearl imaging system. Spatial distributions of both imaging agents in the entire node were compared to confirm their equivalent diffusion kinetics.

Swine cervical lymph nodes (n=3) were used as the control lymph node mode to evaluate the bisected submerge staining protocol. Figure 1 demonstrated that in normal swine lymph nodes, the dynamics of uptake and washout of targeted imaging agent (Aby-029) is similar to control imaging agent (IRDye-700DX carboxylate) ($r=0.99$, $p<0.001$). Both antibody-based imaging agents are capable of penetrating the lymph nodes and then being washed out after the rinsing process. Since the lymph node were bisected and led to a thinner diffusion depth, the internal

fluorescence signals are now more closed to lymph node surface signal as shown in Figure 1(b). The uptake of both imaging agents along the cross-sectioned lymph node confirmed the comparable spatial distribution (r=0.92) and penetration into the lymph node.

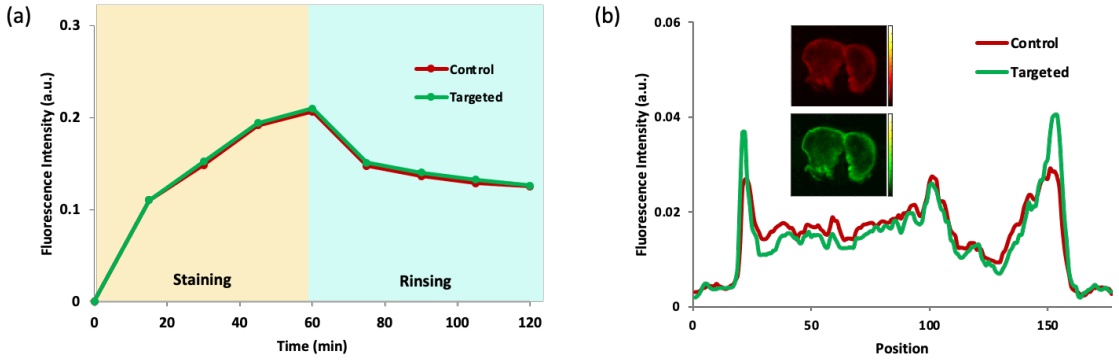

Figure 1. Paired-agent bisected submerge staining and rinsing protocol demonstrated in normal swine lymph node. Lymph nodes were stained in mixture of target and control agent for 1 h and followed by 1 h rinsing with PBS. Fluorescence intensity of whole lymph node for targeted imaging agent (Aby-029) and control imaging agent (IRDye-700DX carboxylate) displayed in (a). Uptake of both imaging agent along the entire cross-sectional lymph node after frozen-sectioned presented in (b).

2.2 Intranodal infusion staining/rinsing protocol evaluation

To further shorten the lengthy process time, we developed an intranodal infusion staining/rinsing protocol for paired-agent imaging approach to detect micrometastases in excised lymph node intraoperatively. An equivalent molar of 2 nM Aby-029 and IRDye-700DX carboxylate was selected as the targeted and control imaging agent, respectively. Lymph node was immersed in saline solution to remain moisture and two 23-gauge butterfly needles (BD Vacutainer blood collection set) was pierced into opposite side of the lymph node. In order to further determine the operational parameters of this intranodal infusion staining approach, three different protocols infusing the same volume of staining and rinsing solution at different infusion rates: where slow group was stained for 10 min and rinsed for 50 min at 30 µL/min infusion rate; moderate group was stained for 5 min and rinsed for 25 min at 60 µL/min; fast group was stained for 1 min then followed by 5 min rinsing at 300 µL/min. The whole lymph node fluorescence signals were acquired and compared after both the staining and rinsing process which were shown in Figure 2 (a) and (b), respectively. Considering the results showed similar uptake and washout among the three protocols, therefore, the fast protocol with 1 min staining followed by 5 min rinsing at 300 µL/min was determined as it provide sufficient infusion in the lymph nodes at most effective parameters.

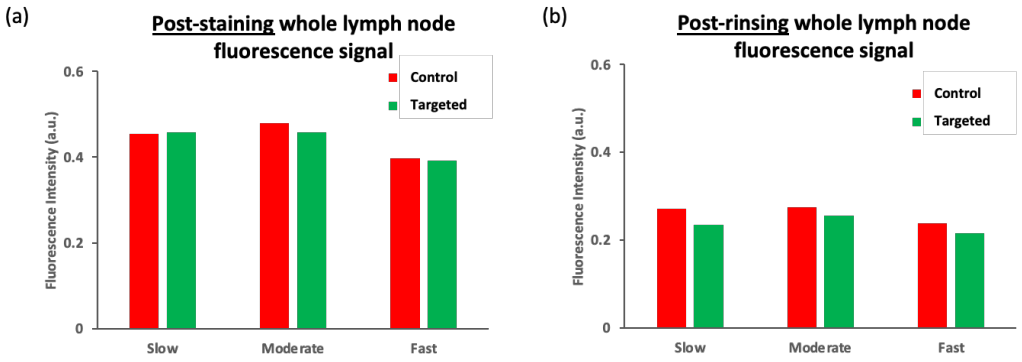

Figure 2. Paired-agent intranodal staining and rinsing protocol comparison with different infusion rate. Whole lymph node fluorescence signal after staining and rinsing process were displayed in (a) and (b), respectively.

Swine cervical lymph nodes (n=3) were pierced by dual-needle then immersed in saline solution and a pre-staining image was acquired by Pearl Imaging System (LICOR Bioscience, Lincoln, NE) to evaluate the background levels caused by autofluorescence. Both targeted and control imaging agent were administrated by infused at a rate of 300 µL/min controlled by a syringe pump for 1min, fluorescence of both imaging agent was recorded immediately after staining. Lymph nodes were then rinsed with PBS at the same infusion rate for 5 min. Images of fluorescence in the targeted and control channel were acquired every 1 min by a wide field fluorescence imaging system (Pearl, LICOR) to compare the concentration of both targeted and control imaging agent. Subsequently, lymph nodes were frozen-sectioning as 200-µm thick slices and imaged under an 85-µm resolution fluorescence imaging system to evaluate the spatial distribution of both agents in the entire lymph nodes.

Figure 3(a) presented the measured time-course fluorescence signal of both imaging agent in excised lymph node during the infusion staining and rinsing process. The results of the uptake and washout kinetics of the targeted and control agent demonstrated a statistically significant correlation with each other in the cancer-free lymph nodes ($r=0.99$, $p<0.001$), with over 70% stained agent being washed out in 5 min. This result demonstrated the suitability of this rapid staining and rinsing protocol using intranodal infusion with Aby-029 as the targeted agent and IRDye700 carboxylate as the control agent. The equivalence of spatial distribution along the lymph node is displayed in Figure 3(b). This result demonstrated that both imaging agents correlated well with each other($r=0.79$) along the lymph node in the absence of binding, suggesting the potential to improve sensitivity of intraoperative micrometastases detection using this intranodal infusion staining/rinsing protocol.

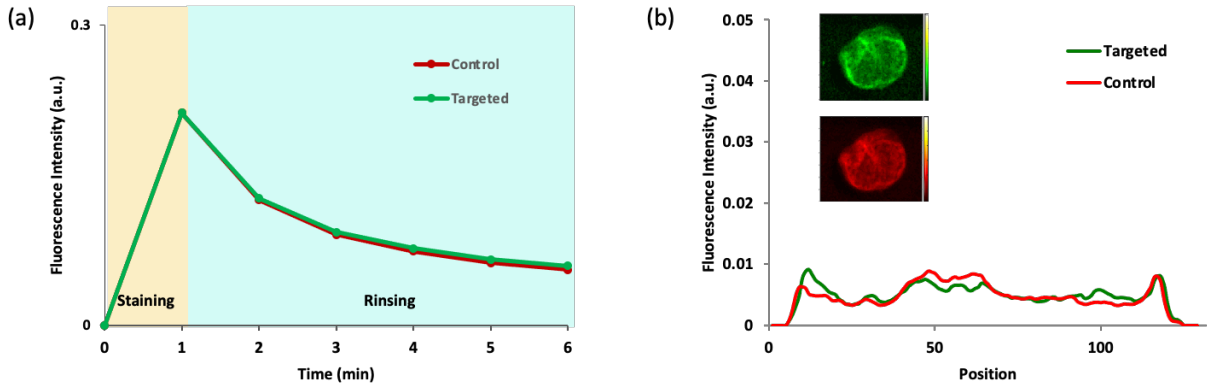

Figure 3. Paired-agent intranodal infusion staining and rinsing protocol demonstrated in normal swine lymph node. Lymph nodes were stained for 1 min followed by 5 min rinse via dual-needle infusion controlled by syringe pump. Fluorescence signal of whole lymph node for targeted imaging agent (Aby-029) and control imaging agent (IRDye-700DX carboxylate) were displayed in (a). Uptake of both imaging agent along the entire lymph node after frozen-sectioned presented in (b).

3. CONCLUSIONS

With equivalent distribution of both imaging agents achieved in the entire lymph node, the paired-agent imaging approach demonstrated its potential for micrometastases detection of intact lymph nodes. While the traditional submerge staining required long process time to stain the entire lymph nodes, the development of intranodal infusion staining protocol allows significant time reduction in staining and rinsing procedure compared to submersion approaches. Further investigation using micrometastatic lymph node model was needed to evaluate the feasibility of this methodology to improve sensitivity of cancer detection of lymph node biopsy intraoperatively.

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