Feasibility of using an enzymatically activatable fluorescence probe for the rapid evaluation of pancreatic tissue obtained using endoscopic ultrasound-guided fine needle aspiration: A pilot study

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Abstract

***Purpose*:** Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is the most reliable method for the histological diagnosis of pancreatic tumors. Rapid on-site fluorescence-guided histological diagnosis was evaluated by topically applying an enzymatically activatable probe onto the EUS-FNA samples; the probe fluoresces in the presence of γ-glutamyltranspeptidase.

***Procedures*:** EUS-FNA was performed in 10 pancreatic tumors. After topical application of the probe, signal intensity was measured using a fluorescence imaging system for 13 min.

***Results*:** In samples from six cases, several regions of the specimens fluoresced and contained adequate tissue for pathological diagnosis. The remaining four non-fluorescent samples contained very small amounts of carcinoma, normal epithelial cells or no epithelial cells. The signal intensity at 5 min was 25.5±7.7 and 7.7±0.5 in fluorescent and non-fluorescent regions, respectively ($p<0.05$).

***Conclusions*:** Application of enzymatically activatable probe onto EUS-FNA samples was feasible for the rapid evaluation of adequate tissues for histological diagnosis.

Keywords: Endoscopic ultrasound-guided fine needle aspiration, Pancreatic tumor, Fluorescent imaging, Enzymatically activatable fluorescence probe, Rapid on-site evaluation

Introduction

Pancreatic cancer is the fourth most common cause of cancer death in men and women [1]. Endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) is a commonly used method for definitive tissue diagnosis of pancreatic cancer with high sensitivity and specificity [2]. In EUS-FNA, however, endoscopists perform a variable number of needle passes to increase the probability of obtaining an adequate sample at the expense of adverse events such as pancreatitis and bleeding [3, 4]. Rapid on-site evaluation during EUS-FNA improves the diagnostic yield, decreasing the number of unsatisfactory samples, and reducing the need for additional passes[5, 6] . However, intensive training is necessary for accurate interpretation of tissue specimens and histological diagnosis [7, 8].

Recently, several target-activatable optical imaging methods have been developed for cancer detection [9-11]. We have previously reported a rapid-cancer detection method involving topical spraying of a γ-glutamyltranspeptidase (GGT)-activated fluorescent probe, γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG) *in vivo*; this probe becomes fluorescent after cleavage of a GGT-specific sequence [12]. The detection of colon cancer using fluorescence after topical administration (spraying) of gGlu-HMRG has been reported in mice and resected human specimens [13, 14].

Here, we applied gGlu-HMRG for the detection of adequate tissue specimens regarding EUS-FNA, and determined the feasibility of this method for rapid on-site detection of pancreatic tumors.

Materials and Methods

Enzymatic-Activatable Fluorescent Probe

The gGlu-HMRG probe was synthesized as previously reported, dissolved in dimethyl sulfoxide (10 mM) and stored at -80°C until used [12]. Briefly, gGlu-HMRG is nonfluorescent under a normal environment without GGT activity. When gGlu-HMRG reacts with GGT on the surface of cancer cells or specimens, it is immediately hydrolyzed and transformed into HMRG, showing strong fluorescence. The gGlu-HMRG probe was dissolved and diluted with phosphate buffered saline (PBS; Life Technologies, Carlsbad, CA, USA) to 1 μM and 10 μM at 1 h before application to cell culture and EUS-FNA samples, respectively.

Cell Culture

The human pancreatic cancer cell lines AsPC-1, PANC-1 and KP4, and rat AR42J exocrine pancreatic tumor cells were used to investigate GGT expression. The AsPC-1 cell line was obtained from Dainippon Sumitomo Pharmaceuticals (Osaka, Japan), and the PANC-1 and AR42J cell lines were obtained from the European Collection of Cell Cultures; they were cultured in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (FBS; Life Technologies), and 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies). The KP4 cell line was obtained from RIKEN BRC through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology, Japan, and cultured in DMEM/F12 (Life Technologies) supplemented with 10% FBS, and 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were cultured in culture flasks in a humidified incubator at 37°C at an atmosphere of 95% air and 5% CO_2.

Fluorescence Microscopy

Cells were cultured in a 35-mm dish and incubated for 24 h. After cells were washed with PBS, 1 μM of gGlu-HMRG in PBS was added and incubated in the dark for 20 min at 37°C. Then fluorescence microscopy was performed using a Biorevo BZ-9000 microscope (Keyence, Osaka, Japan), equipped with the following filters: excitation wavelength, 450–490 nm; and emission wavelength, 500–550 nm. Phase contrast images were also acquired.

Flow Cytometry

Cultured cells were treated with 0.5% trypsin-ethylenediaminetetraacetic acid (Life Technologies), harvested and resuspended in PBS. Cells (1×10^6) were incubated in the dark with 1 μM of gGlu-HMRG in PBS for 20 min at 37°C. Subsequently the cells were analyzed using a flow cytometer (FACSCanto II; Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Endoscopic Ultrasound-guided Fine Needle Pancreatic Tissue Acquisition

Endoscopic ultrasound-guided fine needle tissue acquisition was performed under conscious sedation with intravenous midazolam and fentanyl using linear echoendoscope (GF-UCT 240-AL5: Olympus Ltd., Tokyo, Japan). After confirming that there were no intervening vessels, pancreatic solid lesions were punctured using 22- or 19-gauge needles (Echotip: Cook Medical Inc., Bloomington, IN, USA) followed by 10–20 to-and-fro movements with continuous negative pressure provided by a 10-ml syringe.

On-site Tissue Evaluation using Fluorescence Imaging

After the needle had been withdrawn, the stylet was reintroduced and the specimen was pushed out onto a slide glass. Then, 100 μM of gGlu-HMRG in PBS was applied to the specimen to cover the whole surface. Immediately after the application, the specimen was examined under a handheld fluorescent imaging system (Discovery: INDEC Medical Systems, Santa Clara, CA, USA), which provided captured white light and fluorescent images with 450–490 nm blue excitation light for 13 min. After fluorescence evaluation, the specimen was collected and fixed in 10% formalin for histological analysis. We measured the signal intensity in the fluorescent regions of the specimens and the non-fluorescent specimens using Image J software (National Institutes of Health, Rockville, MD, USA) every 1 min over a period of 13 min.

Histological Analysis

Histological analysis was performed by a single expert pathologist (K.H.), who was blinded to the clinical data, based on hematoxylin and eosin staining. In each sample, the quantity was assessed using the scoring system as previously reported [15]. Briefly, the scoring system was as follows: score 0, insufficient material; score 1, sufficient material for limited cytological interpretation; score 2, sufficient materials for adequate cytological interpretation; score 3, sufficient material for limited histological interpretation; score 4, sufficient material for adequate histological interpretation with low quality; and score 5, sufficient material for adequate histological interpretation with high quality. The proportion of adequate tissue samples was defined as number of adequate tissue samples (adequacy score of ≥3) divided by the total number of tissue samples. Fisher's exact test was applied to the difference in proportion between fluorescent and non-fluorescent regions.

Immunohistochemistry

Immunohistochemistry was performed to examine GGT expression in each EUS-FNA sample. In patients who underwent surgical resection, GGT expression was also evaluated in the resected specimens. Slides were deparaffinized in xylene and sequentially washed in 100%, 95%, 75% and 50% ethanol and subsequently washed in PBS. After heat-induced antigen retrieval (citrate at pH 6), the slides were preincubated in 1% H_2O_2 followed by incubation in blocking solution (normal goat serum) for 2 h. The slide was then incubated in primary anti-GGT1 antibody (Abcam, Cambridge, MA, USA) at 4°C overnight at a dilution of 1:1000, followed by a biotin-conjugated secondary anti-rabbit antibody (Vector Labs, Burlingame, CA, USA) with a dilution of 1:200 to amplify the signal, resulting in greater sensitivity. The slides were then incubated with an ABC reagent (Vectorstain ABC Kit: Vector Labs) for 30 min.

Results

GGT Expression in Pancreatic Cancer Cells

We first evaluated gGlu-HMRG fluorescence in three pancreatic cancer cell lines (AsPC-1, PANC-1 and KP4) and pancreatic acinar cells (AR42J) to determine GGT expression. Using fluorescence microscopy, gGlu-HMRG fluorescence was detected in the AsPC-1, PANC-1 and AR42J cells, but not the KP4 cells (Fig. 1a). Flow cytometric analysis also revealed GGT expression in PANC-1, AsPC-1 and AR42J cells, but not in KP4 cells (Fig. 1b).

Detection of Adequate Tumor Tissue in EUS-FNA Samples using Fluorescence Microscopy

We performed EUS-FNA on 10 patients with pancreatic tumors (Table 1). After gGlu-HMRG was applied topically (sprayed) onto the EUS-FNA samples, several regions of the specimens (six samples) immediately exhibited fluorescence; the remaining four samples

did not exhibit fluorescence over the 13 min observation period. Fluorescent specimens contained adequate tissue for pathological diagnosis and their adequacy score was ≥2. The use of EUS-FNA led to the accurate diagnosis of pancreatic tumor in all of the specimens. Non-fluorescent specimens contained a very small amount of carcinoma, normal epithelial cells, acinar cells or no epithelial cells, and their adequacy score was ≤2. The proportion of adequate tissue materials (adequacy score ≥3) was 83% and 0% in fluorescent and non-fluorescent specimens, respectively (p=0.048). Three representative cases are presented as follows.

Case 4: Pancreatic Adenocarcinoma

EUS-FNA was performed on a 63-year-old male with a pancreatic body tumor (Fig. 2a). Several regions of the specimen exhibited fluorescence immediately after spraying of gGlu-HMRG (Fig. 2b). Histological examination of the fluorescent regions demonstrated that the tissue contained a number of tumor cells expressing GGT (Fig. 2c). In contrast, there were a small number of tumor cells that expressed GGT within the numerous red blood cells in the non-fluorescent regions (Fig. 2d). The histological adequacy score was 5.

Case 6: Focal Pancreatitis

EUS-FNA was undertaken on a 57-year-old female with a pancreatic body tumor (Fig. 3a). After gGlu-HMRG administration, the specimen did not become fluorescent (Fig. 3b). Histological examination of the specimen demonstrated that there was only a small number of epithelial cells without atypia within the numerous red blood cells (Fig. 3c). During the 1-year follow up, the diameter and appearance of this tumor was unchanged. The histological adequacy score was 2.

Case 10: Pancreatic Neuroendocrine Tumor

A 77-year-old female with pancreatic tail tumor underwent EUS-FNA (Fig. 4a). Several parts of the specimen became fluorescent after the spraying of gGlu-HMRG (Fig. 4b). Histological examination of the fluorescent regions demonstrated that the tissue contained atypical cells with oval nuclear and pancreatic acinar cells expressing GGT (Fig. 4c). The patient received distal pancreatectomy and the surgical specimens revealed a neuroendocrine cell tumor that weakly expressed GGT (Fig. 4D). The histological adequacy score was 3.

Fluorescence Intensity of the EUS-FNA Samples

The time-course of the fluorescent images for Cases 4 and 5 are shown in Figure 5a, and the signal intensities in the fluorescent and non-fluorescent regions are shown in Figure 5b. Mean signal intensity in the fluorescent regions was 25.5±7.7 at 5 min after administration. In the six samples with fluorescent regions, architecturally intact pieces of tissue sufficient for histological evaluation of the targeted lesion were confirmed. Histological diagnoses of the EUS-FNA samples were five pancreatic adenocarcinomas and one neuroendocrine tumor (Table 1). In contrast, non-fluorescent samples were not sufficient for histological evaluation, and contained normal epithelial cells or no epithelial cells. In two patients, no adequate tissue samples were obtained. One of these patients had epithelial cells with no atypia, and another had a small amount of atypical cells. The mean signal intensity in the non-fluorescent regions was 7.7±0.5 at 5 min after administration.

Discussion

In the present study, we evaluated a novel technology for the immediate detection of adequate EUS-FNA specimens of pancreatic neoplasm based on an enzymatically activatable fluorescent probe (gGlu-HMRG), which was rapidly activated by GGT. Although GGT was highly expressed in the whole pancreas, especially in the normal acinar cells, the fluorescent regions correlated with adequate specimens of pancreatic tumors leading to accurate diagnosis. This technique could not only minimize the number of needle punctures required without adversely affecting pathological diagnosis, but could also be used in place of rapid on-site evaluation involving cytopathologists.

As described in this study, GGT was expressed in the acini of the normal pancreas as demonstrated using immunohistochemistry. Our *in vitro* study also demonstrated that AR42J rat acinar cells expressed GGT. This was consistent with the findings of previous studies [16-18]. Recently, GGT expression has also been used for the visualization of the leakage of pancreatic juice using gGlu-HMRG [19]. GGT has been reported to be expressed at higher levels in various cancer cells than in normal cells or in inflammation [13, 20]. In the present study, AsPC-1 and PANC-1 cells expressed GGT, but KP4 cells did not. This finding was consistent with previous reports that GGT fluorescent activity was enhanced in approximately 60% of the cultured cancer cells [21]. GGT has been reported to be overexpressed in several human cancer cells and is considered to be a potential biomarker for early cancer detection [22-24]. Recently, topical spraying of gGlu-HMRG has been demonstrated to be useful for the selective fluorescent imaging of colorectal tumors owing to the upregulated GGT activity [14]. Our study demonstrated that pancreatic ductal adenocarcinoma strongly expressed GGT, and that gGlu-HMRG was useful for the detection of adequate specimens of pancreatic cancer. However, immunohistochemical analysis revealed that GGT was not strongly expressed in neuroendocrine tumor cells as compared

with normal acinar cells. Therefore, the effectiveness of gGlu-HMRG regarding the rapid diagnosis of pancreatic neuroendocrine tumors remained undetermined.

In EUS-FNA of pancreatic tumors, adequate specimens are necessary for accurate histological diagnosis [25]. To improve the diagnostic ability of EUS-FNA, rapid on-site evaluation using Diff-Quick staining is performed by a cytopathologist or an endosonographer [5, 26, 27]. However, this procedure costs time and money, and intensive training is necessary for improving accuracy [8, 28]. Recently, use of macroscopic on-site evaluation involving 19-gauge needle EUS-FNA has been reported; however, 19-gauge needle puncture is sometimes difficult, especially for pancreatic head lesions [29, 30]. Using our novel technique, adequate tissue samples obtained using both 19- and 22-gauge needles could be visualized within 5 min after probe application. However, because gGlu-HMRG was not found to be highly specific regarding the detection of pancreatic cancer tissue in EUS-FNA samples, contamination by normal acinar cells could not be excluded. Recently, tumor-specific *in vitro* imaging using adenovirus-mediated gene transfection-induced telomerase promoter-regulated expression of fluorescent proteins has been reported [31, 32]. Although this technique might lead to more specific cancer diagnosis, it is not possible to apply it to rapid on-site cancer diagnosis within 5 min, as is the case with gGlu-HMRG.

The current study had several limitations. The number of patients was relatively small, and use of this probe needs to be evaluated prospectively in studies involving large numbers of patients. In addition, we only evaluated two types of pancreatic tumor, pancreatic ductal adenocarcinoma and neuroendocrine tumor. EUS-FNA is widely used in the histological diagnosis of pancreatic tumors, and lymph node and gastrointestinal submucosal tumors [26, 33, 34]. The effectiveness of novel on-site evaluation regarding other tumor types remains unknown; however, the application of gGlu-HMRG could have a clinical impact concerning the rapid on-site evaluation of EUS-FNA specimens.

Conclusions

Application of enzymatically rapid-activatable fluorescent probe to EUS-FNA samples was found to be a highly reliable method for the immediate detection of adequate tissue materials for evaluation and diagnosis. This technique could minimize the number of needle punctures required, without the expense associated with conventional pathological diagnosis.

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Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: "All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards."

Informed consent: "Informed consent was obtained from all individual participants included in the study."

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immunohistological, flow cytometric, and cytogenetic assessments. Am J Gastroenterol 107:397-404.

Figure Legends

Fig. 1 Fluorescence of pancreatic cell lines *in vitro*. **a** Photomicrographs obtained using fluorescence microscopy showing that γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG) fluorescence can be detected in AsPC-1, PANC-1 and AR42J cells, but not in KP4 cells. **b** Histograms obtained by flow cytometry showing γ-glutamyltranspeptidase (GGT) expression in AsPC-1, PANC-1 and AR42J cells, but not in KP4 cells.

Fig. 2 Fluorescent imaging of an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) sample from Case 4. **a** CT image showing a hypoenhanced tumor on the pancreatic body (left); EUS image showing that the needle was punctured in the hypoechoic tumor (right). **b** Macroscopic image of the EUS-FNA specimen (left); fluorescent image at 13 min after spraying 100 μM of γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG; right). Scale bars = 10 mm. **c** Hematoxylin and eosin staining of the EUS-FNA specimen from the fluorescent region (arrow to the right of **b**; left); immunohistochemical staining showing that γ-glutamyltranspeptidase (GGT) was expressed in the ductal carcinoma (right). Scale bars = 500 μm. **d.** Hematoxylin and eosin staining of the EUS-FNA specimen from the non-fluorescent region (arrowhead to the right; left); immunohistochemical staining showing that the small amount of cancer cells expressing GGT (right). Scale bars = 100 μm.

Fig. 3 Fluorescent imaging of an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) sample from Case 6. **a** CT image showing a hypoenhanced tumor on the pancreatic body (arrow; left); EUS image showing that the needle punctured the hypoechoic tumor (right). **b** Macroscopic image of the EUS-FNA specimen (left); fluorescent image at 13 min after spraying 100 μM of γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG; right). Scale bars = 10 mm. **c** Hematoxylin and eosin staining of the EUS-FNA specimen

(left); immunohistochemical staining showing that a small number of the epithelial cells expressed γ-glutamyltranspeptidase (GGT; right). Scale bars = 500 μm.

Fig. 4 Fluorescent imaging of an endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) sample from Case 10. **a** CT image showing a hyperenhanced tumor on the pancreatic tail (arrow; left); EUS image showing the hypoechoic tumor (arrow; right). **b** Macroscopic image of the EUS-FNA specimen (left); fluorescent image at 13 min after spraying 100 μM of γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG) (right). Scale bars = 10 mm. **c** Hematoxylin and eosin staining of the EUS-FNA specimen (left); immunohistochemical staining showing that γ-glutamyltranspeptidase (GGT) is expressed in the neuroendocrine tumor and acinar cells (right). Scale bars = 100 μm. **d** Hematoxylin and eosin staining of the surgical specimen (left); immunohistochemical staining showing that GGT is expressed in the tumor neuroendocrine and acinar cells (right). Scale bars = 500 μm.

Fig. 5 Longitudinal course of signal intensity in endoscopic ultrasound-guided fine needle aspiration (EUS-FNA) specimens. **a** Time-course of the fluorescent imaging of the EUS-FNA specimens at 0, 2, 5, 9 and 13 min after spraying on γ-glutamyl hydroxymethyl rhodamine green (gGlu-HMRG; Cases 4 and 5).

b Signal intensity in the fluorescent and non-fluorescent regions of the specimens from the six cases.

Table 1. Tumor characteristics

	Location	Diameter (mm)	Needle (gauge)	Histology	Adequacy*	Fluorescence intensity[#]	Final diagnosis
1	Head	33.7	22	Adenocarcinoma	3	118	Adenocarcinoma
2	Head	10	22	Insufficient materials	0	NA	Focal pancreatitis
3	Body	12	22	Atypical gland	1	NA	Adenocarcinoma
4	Body	32.5	19	Adenocarcinoma	5	66	Adenocarcinoma
5	Head	11.4	22	Adenocarcinoma	4	55	Adenocarcinoma
6	Body	13.7	22	Normal epithelium	2	NA	Focal pancreatitis
7	Head	46	22	Adenocarcinoma	2	18	Adenocarcinoma
8	Head	28.6	22	Adenocarcinoma	3	27	Adenocarcinoma
9	Tail	20.6	22	Insufficient materials	1	NA	Adenocarcinoma
10	Tail	12.5	22	Atypical cells	3	27	Neuroendocrine tumor

* The adequacy score was calculated as detailed in a previous report [15].

[#] Signal intensity of the fluorescent region at 13 min. NA; not analyzed

Figure 1A

Figure 1

A

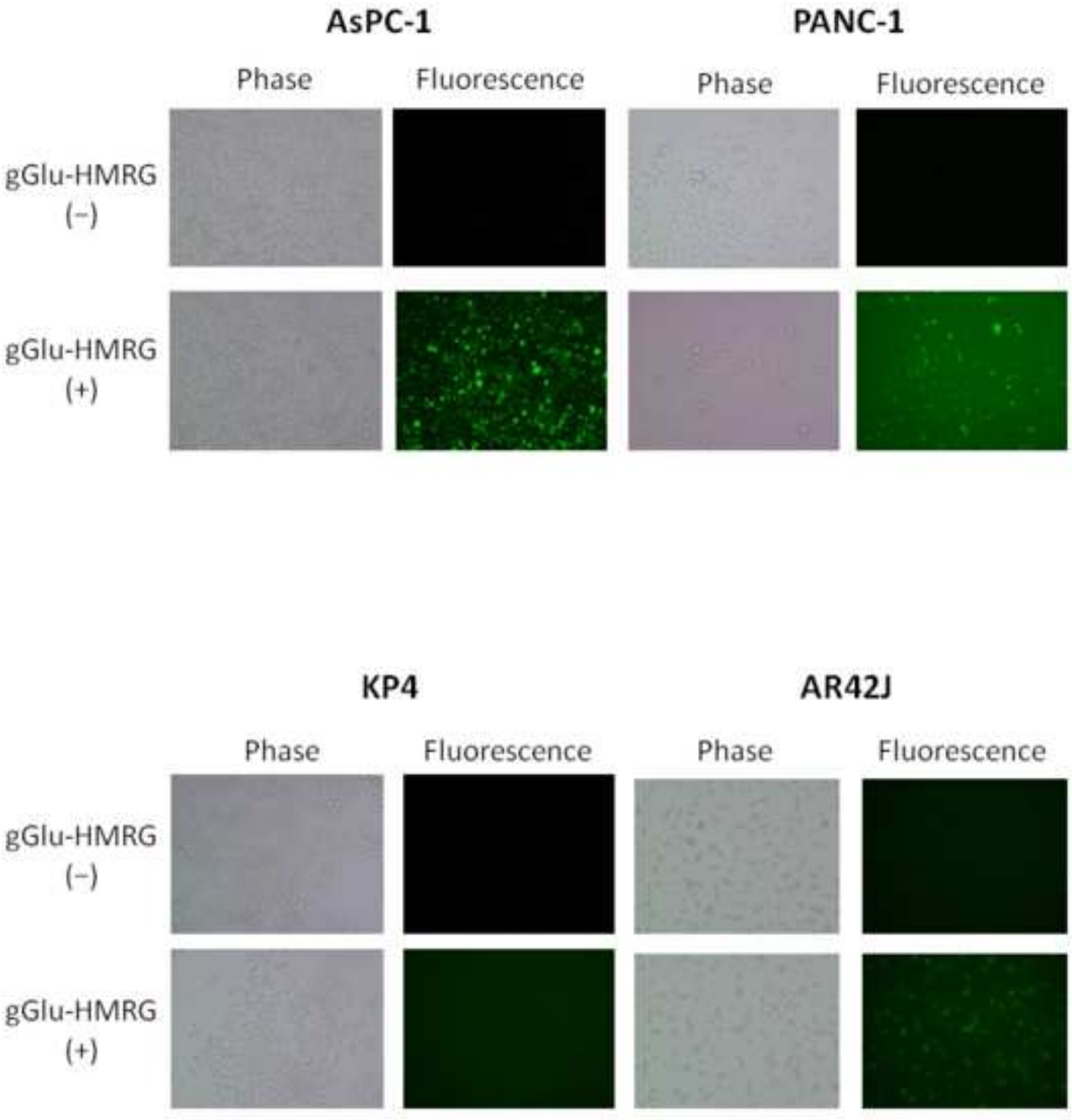

Figure 1B

Figure 1

B

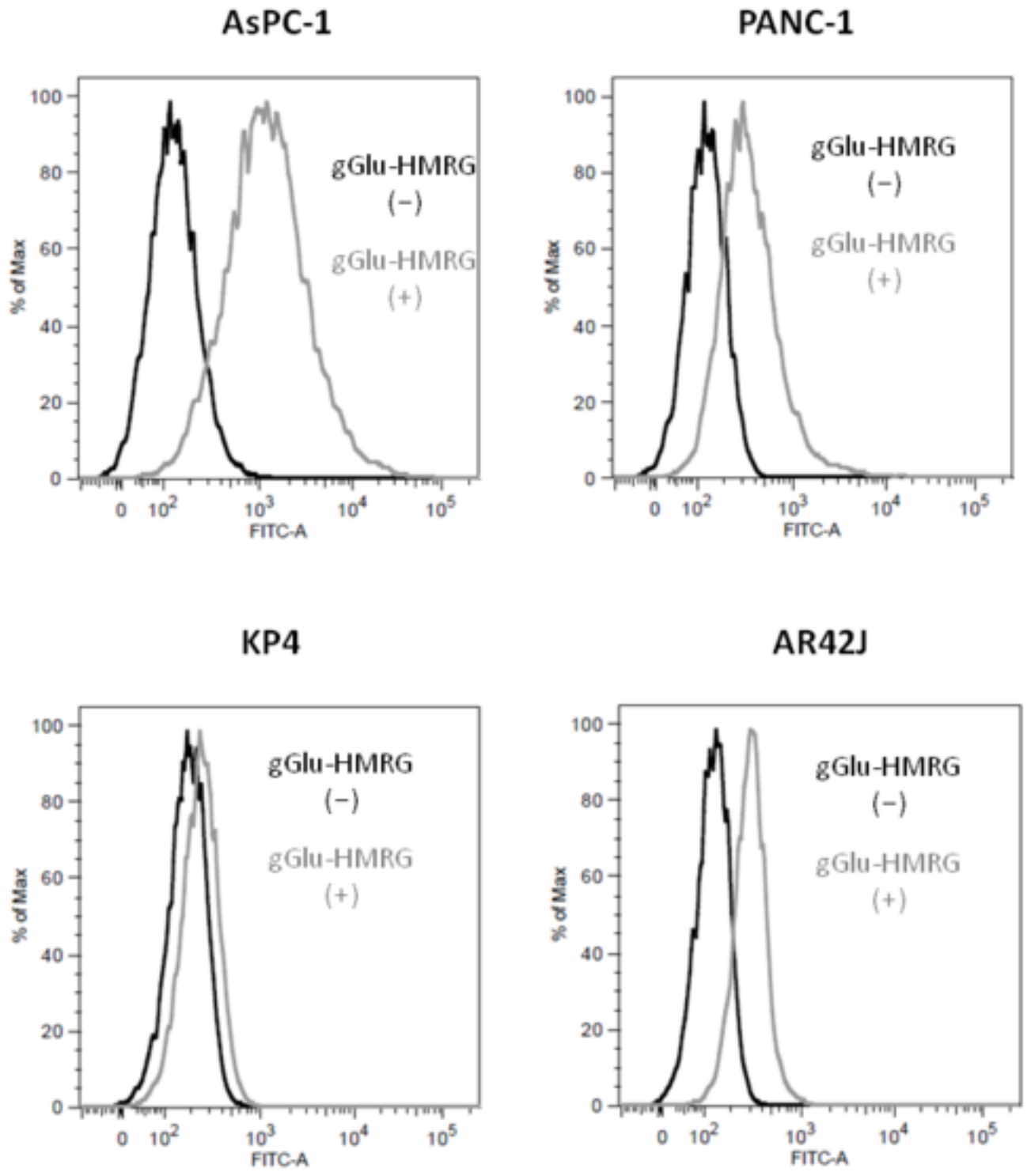

Figure 2

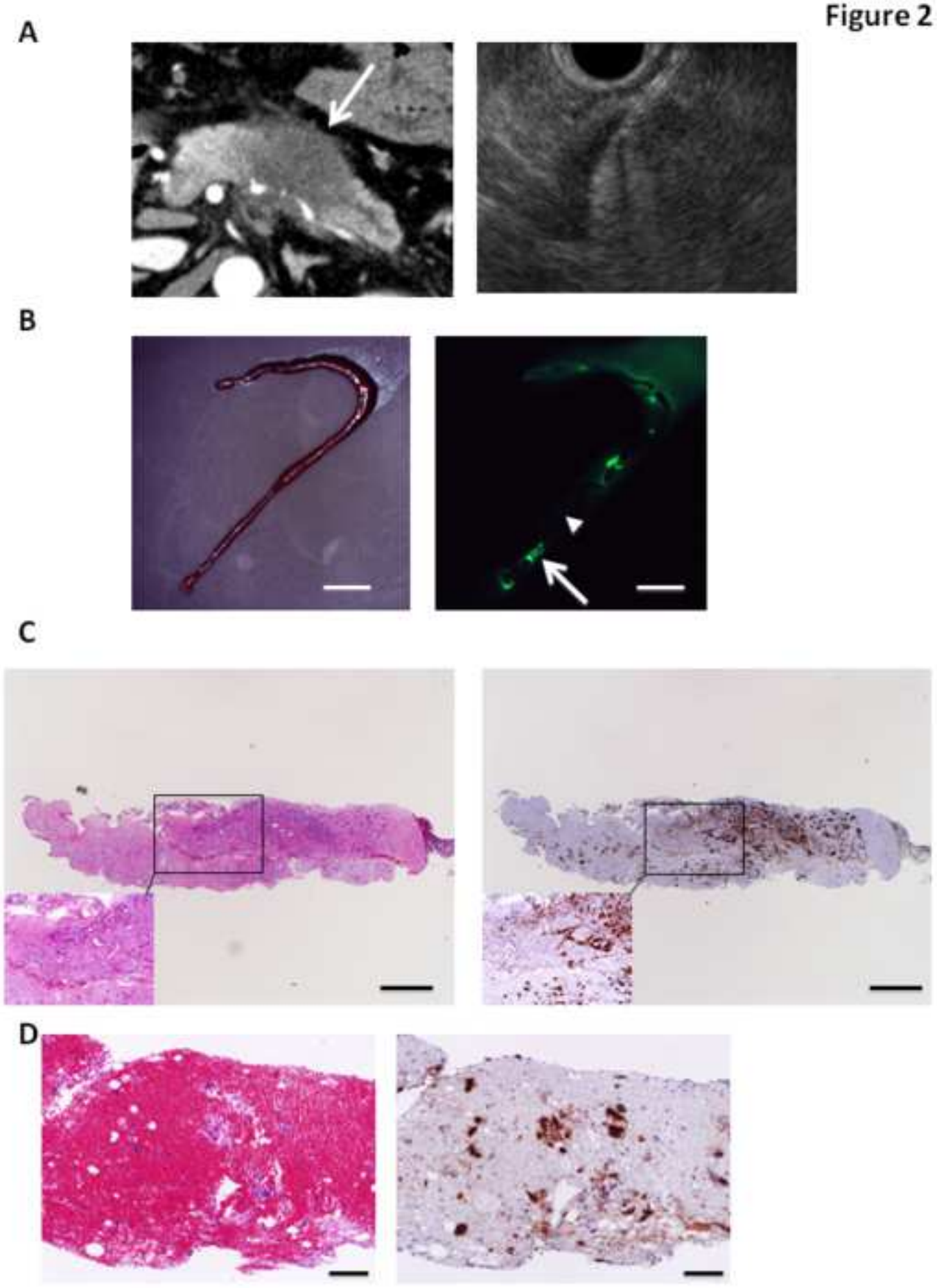

Figure 3

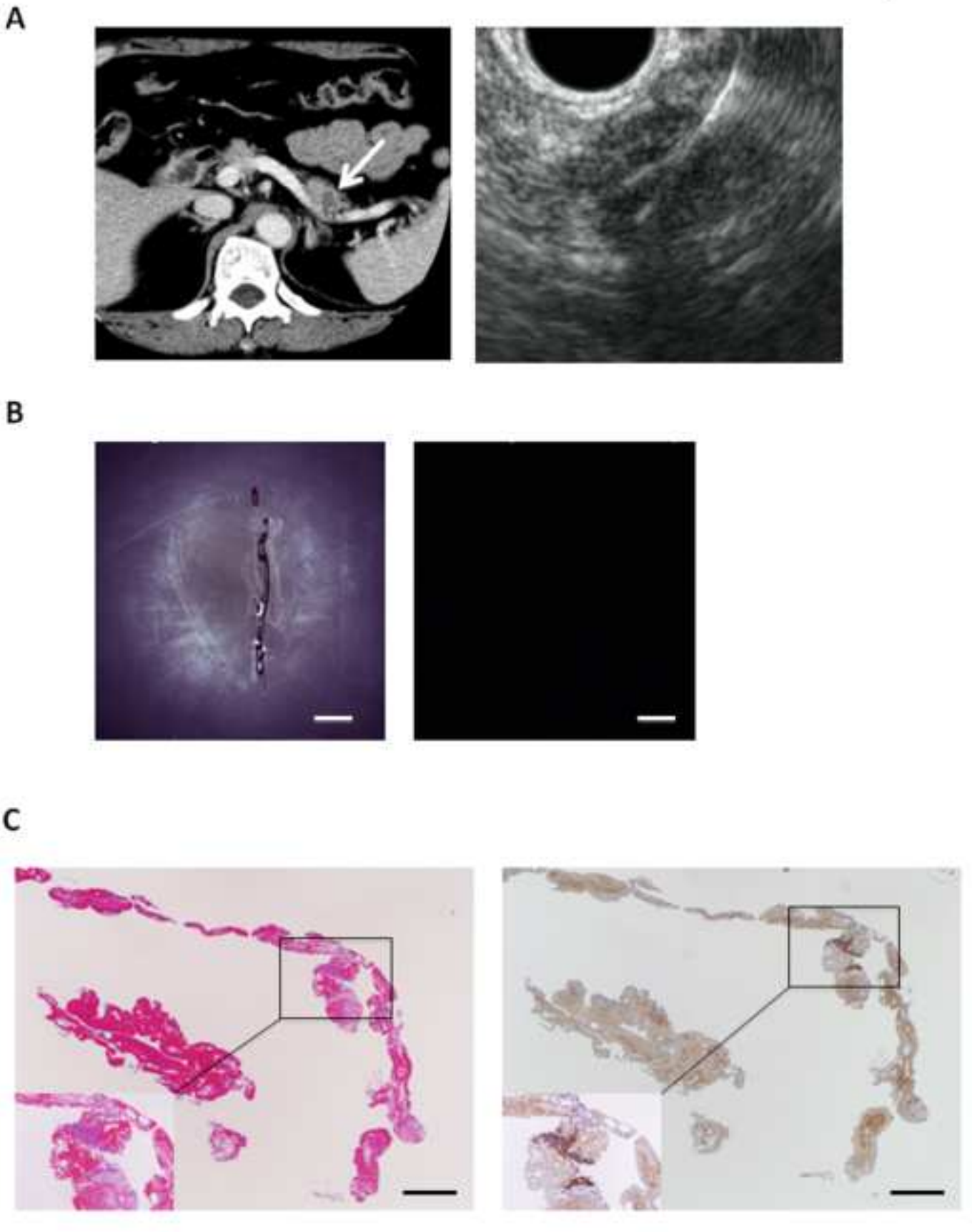

Figure 4

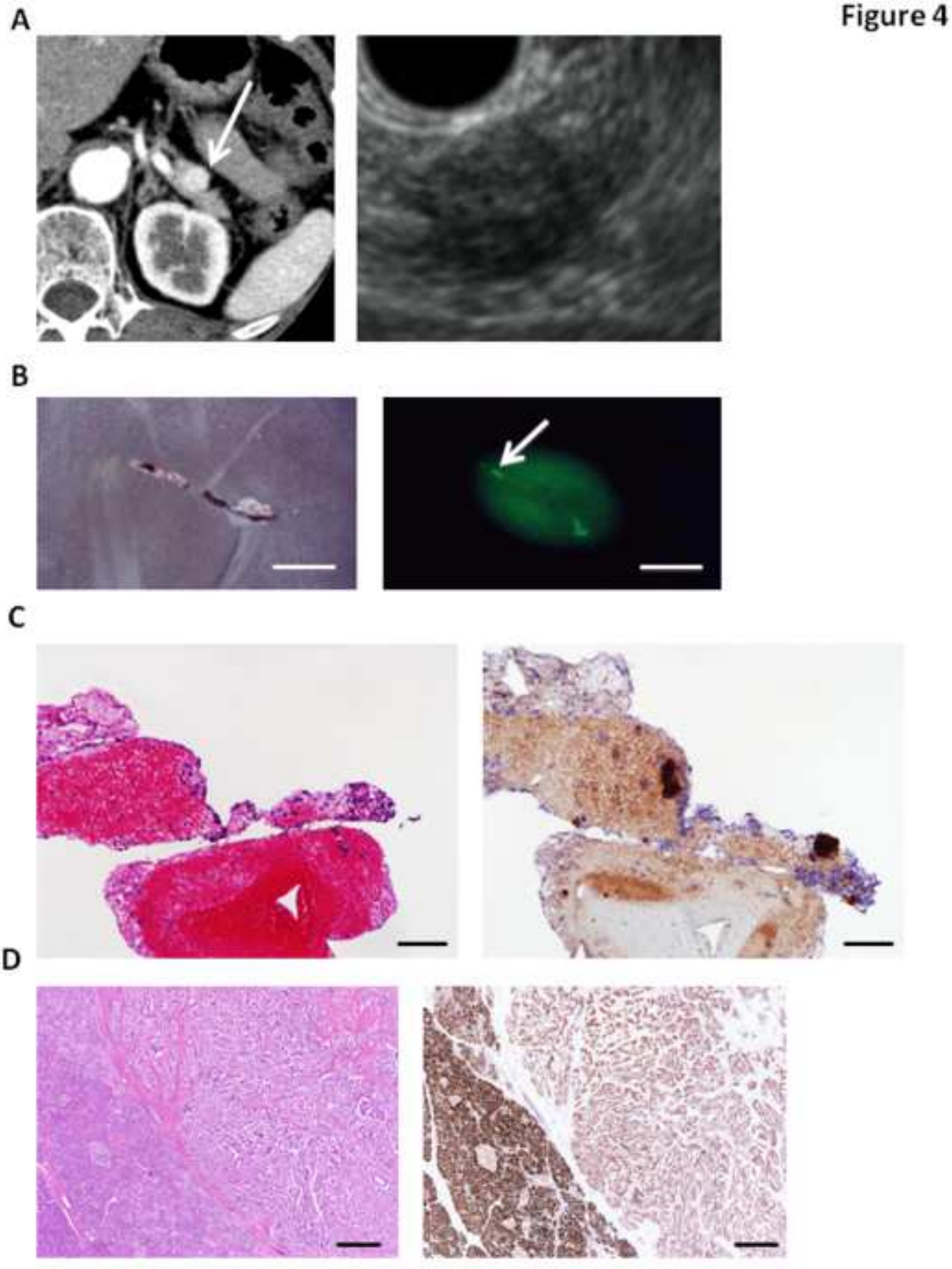

Figure 5

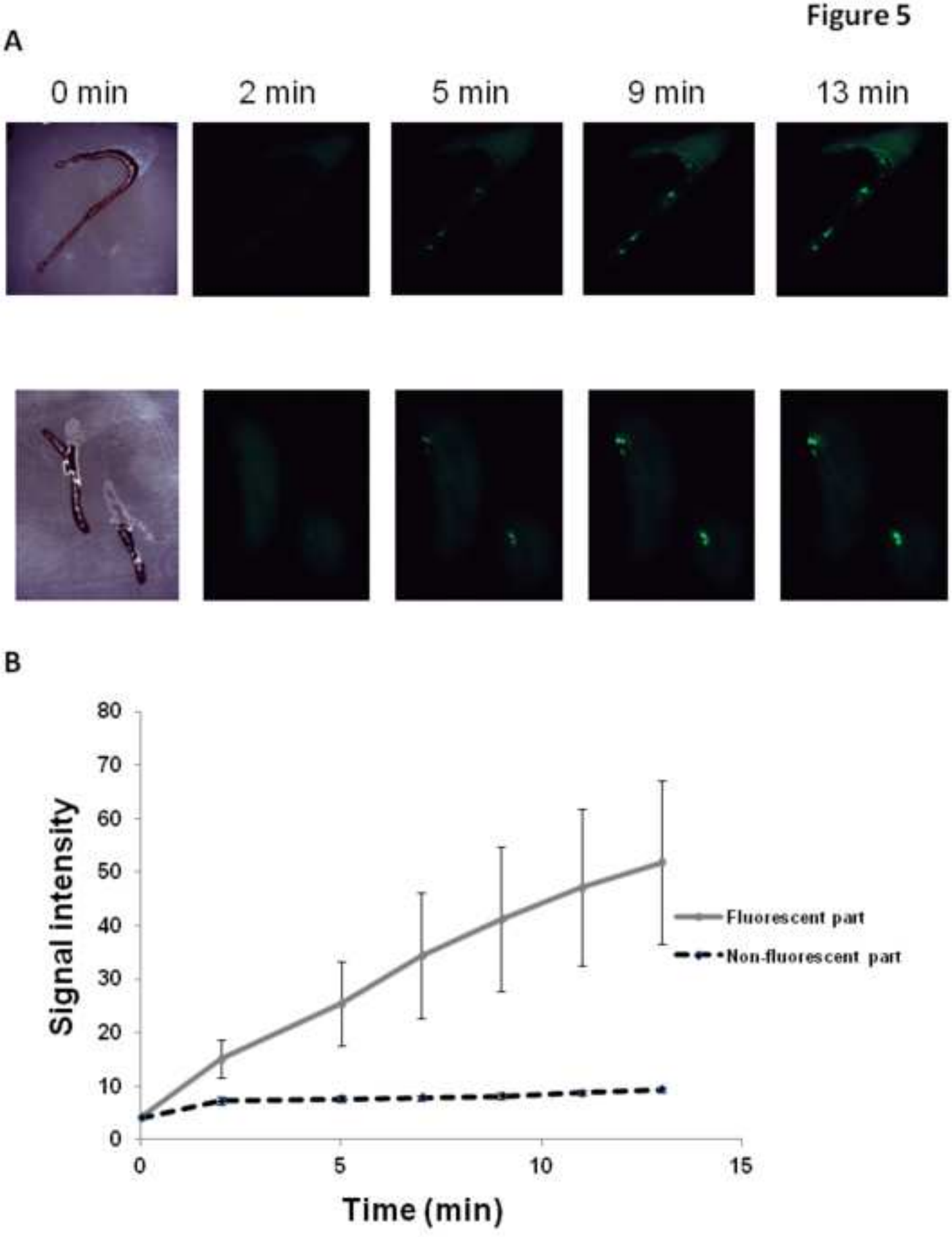
Figure 5
A
0 min
2 min
5 min
9 min
13 min
B
Signal intensity
80
70
60
50
40
30
20
10
0
0
5
10
15
Time (min)
Fluorescent part
Non-fluorescent part