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*Original Article*

# Comparison of immunofluorescence and immunohistochemical staining with anti-insulin antibodies on formalin-fixed paraffin-embedded human pancreatic tissue microarray sections

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**Abstract:** Immunohistochemistry (IHC) and immunofluorescence (IF) are two most often used tools in the researches of tissue morphology and cell biology. In order to demonstrate which is more sensitive in the detection in paraffin embedded tissues (PET), we compared the detection rate of these two methods with human pancreatic tissue microarrays (TMA) by using three anti-human insulin antibodies. The results showed that the detection rates are mostly depending on the primary antibody (Ab) chosen. For some primary Abs, there were no significant differences between IHC and IF in PET tissues, such as primary Ab No. 1 (85.7%, 18/21 vs 76.2%, 16/21) and Ab No. 3 (95.2%, 20/21 vs 95.2, 20/21). In contrast, for other primary Abs (No. 2), the detection rate of IF is much lower than that of IHC in PET tissues (38.1%, 8/21 vs 90.5%, 19/21). Interestingly, the detection rate of IF was increased after the same sections were re-stained with the procedure of IHC. In addition, our results also showed that IHC method can be executable after IF method was finished in the same PET tissues, while it is hardly to execute IF after the DAB staining of IHC method was completed. We conclude that whether IF or IHC method can be applicable in the PET tissues largely depend on the primary Abs you have chosen. Also, we found that IHC method can still be executable after IF method was finished in the same PET tissues.

**Keywords:** Immunofluorescence, immunohistochemistry, anti-insulin antibody, human pancreatic tissues, paraffin embedded tissue

## Introduction

Immunohistochemistry (IHC) and immunofluorescence (IF) are two powerful investigational tools in the tissue morphology and cell biology researches [1, 2]. IHC is a method for demonstrating the presence and localization of antigens (usually proteins) in tissue sections and cells by the use of an antibody (antibodies) with specificity for an antigen (antigens). These are subsequently visualized by a marker such as an enzyme forming a color precipitate (such as 3', 3'-diaminobenzidine (DAB) et al). IHC staining is now a main ancillary technique used in the clinical disease diagnosis, especially in paraffin embedded tissues (PET) [3].

IF method is a specialized type of IHC, which uses a fluorescent dye to visualize antibody binding under a fluorescent microscopy. The commonly used fluorescent dyes include fluorescein isothiocyanate (FITC), TRITC (tetraethylrhodamine isothiocyanate) or Rhodamine. There are two major types of IF staining methods: Direct method and indirect method. Indirect immunofluorescence (IIF) is often applied for its high sensitivity, and it is mostly used in the living cells or fresh tissues, seldom in PET tissues, partially because of the high fluorescent background [4], but the definite reason is still unclear.

The current studies was designed to evaluate the sensitivity and specificity of the above IHC and IIF methods by employing human pancreatic PET tissue microarrays (TMA) with three types of anti-human insulin antibodies. And further to explore the possible factors to enhance the affect the IIF.

**Table 1.** Antibodies and agents used in the present study

| Abs | Abbre | Isotype control | Reactivity | Expression/ Localization | Dilution | Sources | Cat No. |
|---|---|---|---|---|---|---|---|
| Primary Ab | No. 1: R-h | IgG | βcells | cytoplasm | 1:100 | Cell signaling | 4590 |
| | No. 2: M-h1 | IgG1 | βcells | cytoplasm | Pre-diluted | Zymed | ZM-0155 |
| | No. 3: M-h2 | IgG1 | βcells | cytoplasm | 1:200 | Abcam | Ab46707 |
| Secondary Ab | G-r-F | IgG | Rabbit | - | 1:100 | CWBIO | CW0114 |
| | G-m-F | IgG | Mouse | - | 1:100 | CWBIO | CW0113 |
| | G-m-H | - | Rabbit | | Pre-diluted | Gold Bridge Inc. | PV-6001 |
| | G-r-H | - | Mouse | - | Pre-diluted | Gold Bridge Inc. | PV-6002 |

Ab: Antibiody; R-h: Rabbit anti-human insulin; M-h: Mouse anti-human insulin; G-r-F: Goat anti-Rabbit IgG FITC conjugate; G-m-F: Goat anti-Mouse IgG FITC conjugate; G-m-H: Goat anti-mouse Polink-1 staining kit (HRP); G-r-H: Goat-rabbit Polink-1 staining kit (HRP), DAB, indicates 3,3'-diaminobenzi dine; HRP: horseradish peroxidase.

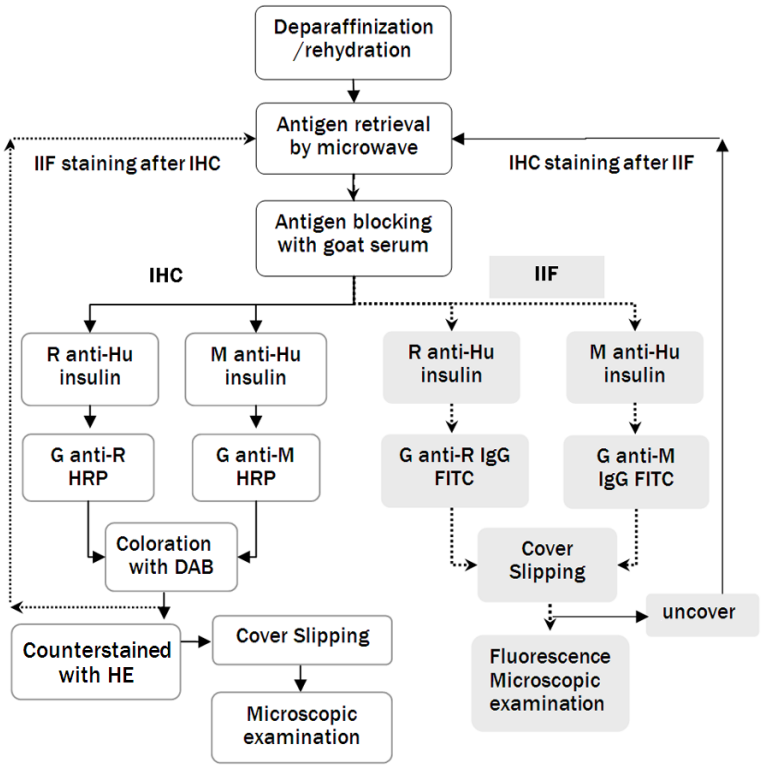


**Figure 1.** Procedures of immunohistochemistry (IHC) and immunofluorescent (IF) staining in the study. Full line (-) is the procedure of IHC and dash line (---) is the procedure of IIF. Note: IHC: Immunohistochemistry; IIF: Indirect immunofluorescent; R: Rabbit; G: Goat; Mouse; Hu: Human; HRP: Horseradish peroxidase; HE: Hematoxylin stain; DAB: 3,3-diaminobenzidin; FITC: Fuorescein isothiocyanate.

## Material and methods

*Tissues and tissue microarrays*

All tissues come from department of pathology Shaanxi Provincial People's Hospital, fixed with buffered formalin for at least 48 hrs, embedded into paraffin blocks. All procedures were in accordance with national guidelines for organ donation and the Hospital of Review Board. The human pancreatic TMA blocks were manufactured in Shaanxi Chaoying Biotechnology Co., Ltd. which includes 21 normal human pancreas tissues; Each tissue represents one individual sample. Four millimeter sections were used for immunostaining.

*Antibodies*

Primary and secondary antibodies used for IHC or IIF are listed in **Table 1**. Briefly, primary antibodies: Rabbit anti-human insulin antibody (#45-90, Cell signaling, Ab No. 1); Mouse anti-human insulin antibody (ZM-0155, IgG, Beijing Zhongshan Golden Bridge Int. Inco. Ab No. 2); (Ab-46707, Abcom Int. Inco, Ab No. 3); Secondary antibodies for IIF: Goat anti-Rabbit IgG FITC conjugates (CW0114, CWBio, Beijing, China); Goat anti-Mouse IgG (H+L) FITC conjugate; Second antibodies for IHC: PV6001 is an anti-Rabbit antibody HRP conjugate; PV6002 is an anti-mouse antibody HRP conjugate (Gold Bridge Int. Inco, GBI, Beijing, China).

*Methods*

*Slide preparation:* Four-μm sections of pancreas TMA were cut and affixed onto glass slides. For removal of paraffin, the slides were

**Table 2.** Comparison of detection rate of IHC with three different primary insulin antibodies

| Abs | | No. 2 anti-insulin | | Total (%) | No. 3 anti-insulin | | Total (%) |
|---|---|---|---|---|---|---|---|
| | | - | + | | - | + | |
| No. 1 anti-insulin | - | 1 | 2 | 3 | 1 | 2 | 2 |
| | + | 1 | 17 | 18 (85.7) | 0 | 18 | 18 (85.7) |
| Total (%) | | 1 | 19 (90.5) | 21# | 1 | 20 (95.2) | 21# |
| No. 3 anti-insulin | - | 0 | 1 | 1 | | | |
| | + | 2 | 18 | 20 (95.2) | | | |
| Total (%) | | 2 | 19 (90.5) | 21# | | | |

Note: Abs: Antibodies; NcNemar Test: #P>0.05.

**Table 3.** Comparison of detection rate of IIF with three different primary insulin antibodies

| Abs | | No. 2 anti-insulin | | Total (%) | No. 3 anti-insulin | | Total (%) |
|---|---|---|---|---|---|---|---|
| | | - | + | | - | + | |
| No. 1 anti-insulin | - | 4 | 1 | 5 | 0 | 5 | 5 |
| | + | 9 | 7 | 16 (76.2) | 1 | 15 | 16 (76.2) |
| Total (%) | | 13 | 8 (38.1) | 21* | 1 | 20 (95.2) | 21## |
| No. 3 anti-insulin | - | 1 | 0 | 1 | | | |
| | + | 12 | 8 | 20 (95.2) | | | |
| Total (%) | | 13 | 8 (38.1) | 21** | | | |

Note: Abs: Antibodies; NcNemar Test: *P<0.05; ##P>0.05; **P<0.01.

immersed in xylene (10 min × 4 times), followed by graded ethanol for 10 min (in 100% × 2 times, 95%, 90%, 85% ethanol respectively). Rinse under gently running water. And then tissues were blocked with 3% $H_2O_2$-methanol for 30 min at room temperature for endogenous peroxidase ablation. All following steps were carried out in a moist chamber.

*Antigen retrieval by microwave method:* The TMAs were placed in the microwaveable vessel and immersed with sodium citrate buffer (pH 6.0). Place the vessel inside the microwave. Set to full power for 2 min, and then mid power for 8 min. And then, slides were allowed to gradually cool in the same buffer after heating.

*Immunohistochemistry (IHC):* The tissue sections were rinsed (5 min × 3 times) with PBS (0.01 M PBS pH 7.4: $KH_2PO_4$ 0.02%, $Na_2HPO_4$ 0.29%, KCl 0.02%, 0.8% NaCl), and wiped off excess buffer and draw a circle around the tissue with a PAP pen. Slides were blocked by incubation in 5% goat serum for 1 hr at 37°C. After discarding the blocking serum, the diluted primary antibodies (details in **Table 1**) were dropped on the sections, and incubate overnight at 4°C in the refrigerator. After incubation for 1 hr at room temperature, the slices were rinsed in PBS (5 min × 3 times). Rabbit secondary antibody (Polink-1 staining kit in **Table 1**) were added, and incubating the sections for hrs at 37°C. After rinsing in PBS (5 min × 3 times), the slices were colorized with 3,3-diaminobenzidin (DAB), kept at room temperature without light for 5 min, and finishing coloration with the distilled water. The sections were counterstained with Mayer's Hematoxylin solution (3 min), thoroughly rinsed with DW, dehydrated to xylene, permanently mounted. The negative control was carried out with the same steps as above, but the primary antibody was replaced by PBS. All incubations should be carried out in a humidified chamber to avoid drying of the tissue (**Figure 1**).

*Indirect Immunofluorescence (IIF):* Immunofluorescence on paraffin sections was carried out with the same steps as IHC procedure on paraffin sections, but the Goat anti-Rabbit IgG FITC conjugate/Goat anti-Mouse IgG (H+L) FITC conjugate was replaced by the Rabbit Polink-1 staining kit (HRP)/Mouse Polink-1 staining kit (HRP). Besides, the slices were covered with 50% glycerin before observed under microscope (**Figure 1**).

*Statistical methods*

The results of IHC and IIF were observed and photographed under a fluorescence microscope Olympus BX41, Shanghai, China). The criteria used to determine positive staining were established by locating the brown/Green staining of islet cells compared to the surrounding pancreatic tissues. Of the 21 TMA pancreatic tissues, all the cases were collected and analyzed with the SPSS software (version 20.0, SPSS Inc. Chicago, IL, USA) and GraphPad Prism software (version 6.02, La Jolla, CA, USA). All *P*-values were two-tailed and values less

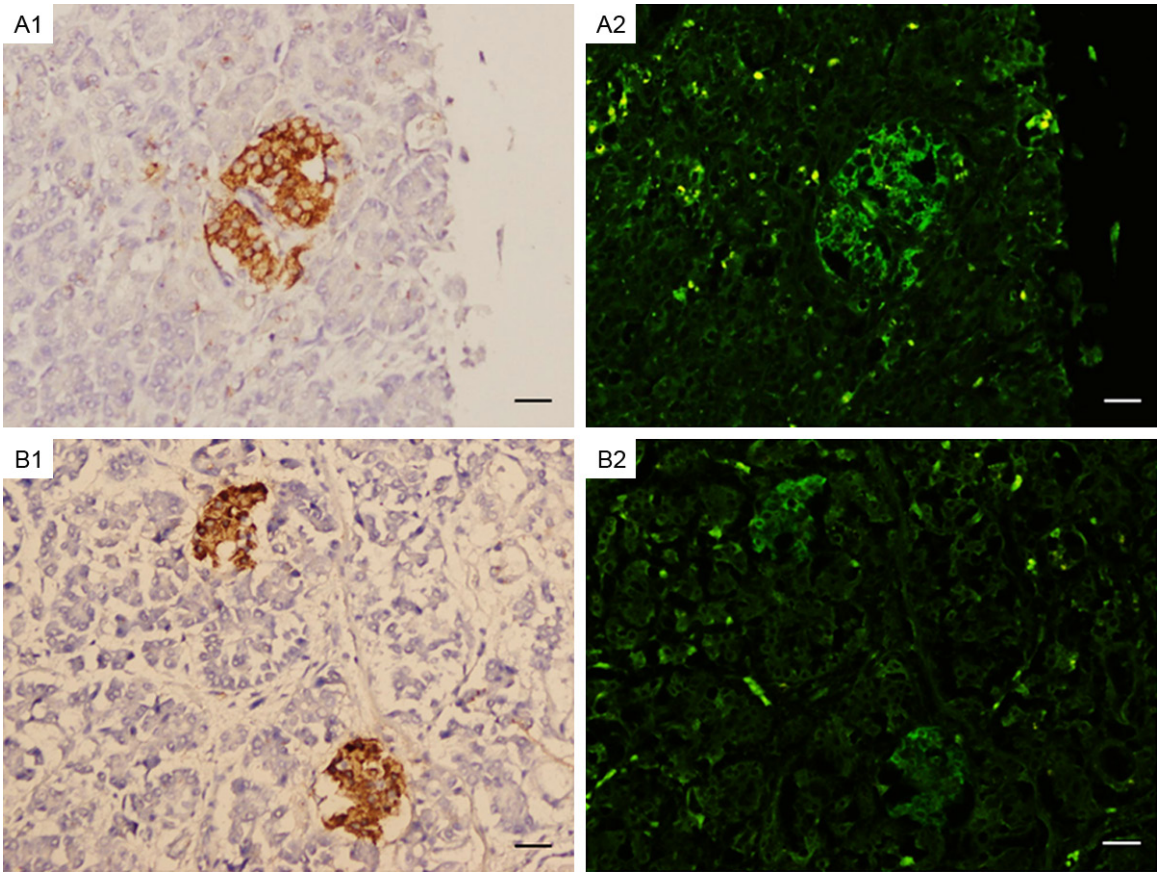


**Figure 2.** Representive images of IHC and IIF staining on human pancreatic TMA. Rabbit anti-human insulin (No. 1) was used as primary antibody in IHC (A1) and IIF (A2) staining. Mouse anti-human insulin 1 (No. 2) was used as primary antibody in IHC (B1) and IIF (B2) staining (bar: 30 μm).

**Table 4.** Comparison of IIF and IHC methods in TMA pancreatic PET tissue with three kinds of anti-insulin Ab

| Methods | | IFF | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Ab. No. 1 | | Total | Ab. No. 2 | | Total | Ab. No. 3 | | Total |
| | | - | + | | - | + | | - | + | |
| IHC | - | 3 | 0 | 3 | 2 | 0 | 3 | 0 | 1 | 1 |
| | + | 2 | 16 | 16 | 11 | 8 | 19 | 1 | 19 | 20 |
| Total | | 5 | 16 | 21# | 13 | 8 | 21** | 1 | 20 | 21# |

NcNemar Test: #P>0.05; **P<0.01; #P>0.05.

than 0.05 were considered statistically significant.

## Results

*Comparison of positive rate of IHC and IIF methods with three primary antibodies*

We firstly compared the detection rate of IHC or IIF staining with three primary antibodies including rabbit anti-human insulin (Ab. No. 1) and mouse-anti-human insulin (Ab. No. 2 and No. 3) (**Tables 2** and **3**). The results showed that there was no significant difference between three antibodies (primary antibody: No. 1: 85.7%, 18/21; No. 2: 90.5%, 19/21; No. 3: 95.2% 20/21) in the IHC detection (**Table 2**). In contrast, the results of IIF showed that the detection rate of primary Ab. No. 2 is much lower than that of Ab. No. 1 and Ab. No. 3 (33.3% 16/21 vs 76.2%, 7/21), but these is no difference between Ab. No. 1 and Ab. No. 3 in IIF detection (**Table 3**).

*Comparison of detection rate of IHC and IFF with same primary antibodies on the same TMAs*

Next, the detection rate of IHC and IFF methods with three kinds of anti-human insulin antibodies is also compared, one is rabbit derived anti-insulin Ab (No. 1), and another is mouse derived

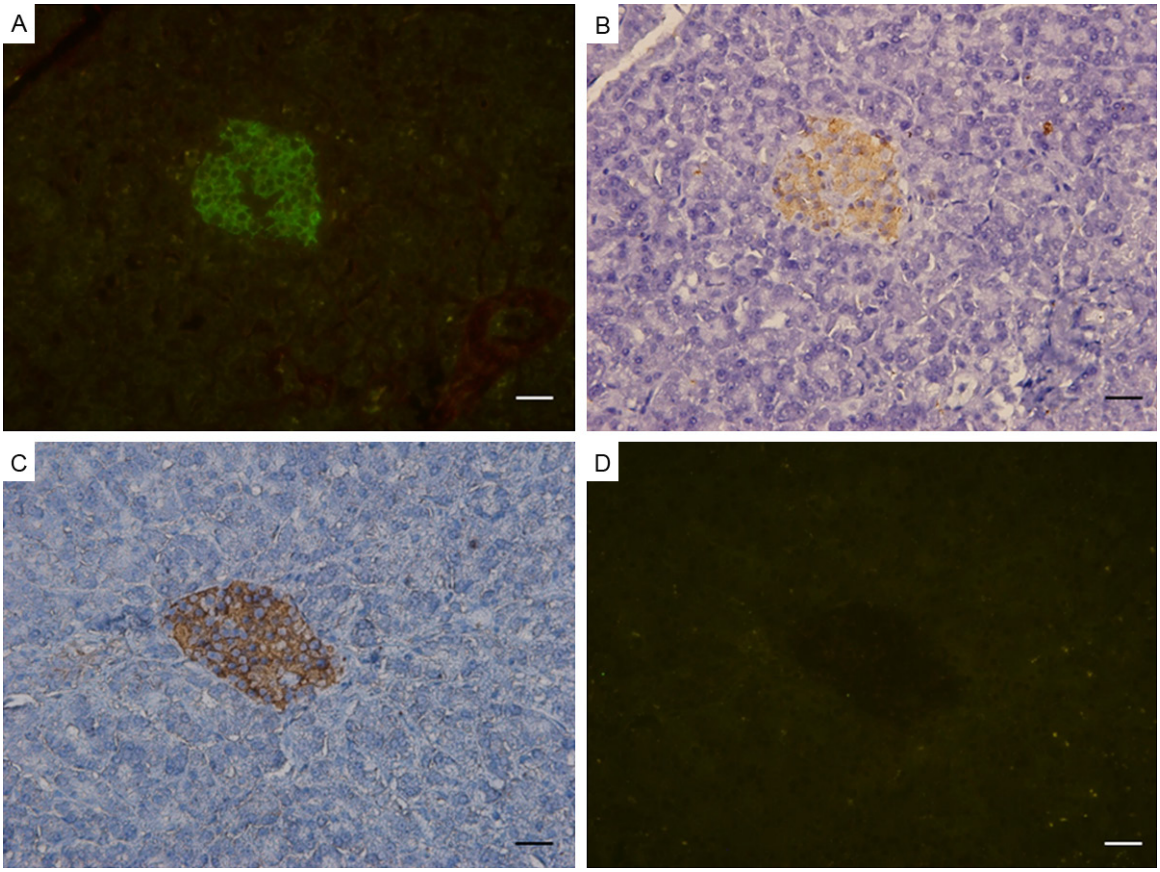


**Figure 3.** Representive image of IHC staining after IFF and IFF staining after IHC with the rabbit anti-human insulin on human pancreatic TMA. A: IIF staining; B: IHC results after IIF staining on the same tissue. C: IHC staining; D: IIF staining results after IHC on the same tissue (bar: 30 μm).

anti-insulin Ab (No. 2). The results showed that there is no significant difference between the detection rate of IHC (85.7%, 18/21) and IIF (76.2%, 16/21) methods with Ab. No. 1 and No. 3 (**Table 3**, **Figure 2A1**, **2A2**), although the detection rates of IFF are slightly lower than those of IHC. In contrast, with another mouse derived antibody (Ab. No. 2), the detection rate of IIF method (33.3%, 7/21) is much lower than that of IHC (95.2%, 20/21) method (**Tables 3** and **4**, **Figure 2B1**, **2B2**).

*The results of IHC staining after IIF and IIF staining after IHC with the same primary Ab on the same TMAs*

Last, we investigated the possibility that will IHC staining be still working after IIF staining on the PET tissues? Similarly, will IIF be still working after IHC staining on the PET tissues (experimental procedures are shown in **Figure 1**)? Observing the results, we found that IHC staining could be observed after IIF staining was performed. In contrast, after IHC staining, IFF staining will be hard to be seen in the detection (**Figure 3**).

## Discussion

In this study, we have compared two mostly used methods IHC and IIF with three different primary antibodies on human pancreatic TMA after the staining conditions were optimized. The results showed that the detection rate of IHC and IIF in PET tissue is mostly depending on the primary antibodies employed. Some antibodies (such as anti-insulin Ab. No. 1 and No. 3 in this study) are fit for the IHC and IFF methods, some antibodies (such as anti-insulin Ab. No. 2) are only suitable for the IHC, not for the IFF method. So it is very important for the users to read the instruction of the antibody before use, especially in PET tissue.

The reason why some antibodies are not fit for the PET tissue is not clear, mostly attribute to the following reasons: The first maybe the poor quality of PET tissues. As we known, archive

PET tissues are often fixed and stored under a variety of sub-optimal conditions, including the use of unbuffered fixation methods and storage under non-climate-controlled conditions, resulting in the degradation or loss of antibody epitopes of these antigen proteins [4]. Numerous methods have been developed to enhance contrast or to enhance tissue reactivity with an antibody via antigen retrieval in IHC [5] and IFF method [6]. In this study, we used microwave antigen retrieval method to overcome these obstacles.

The second reason may be the different epitopes of antibodies. There would be many antibodies against the same antigen (such as human insulin antigen). One antibody may against only one epitope of the whole antigen. Some epitope maybe easily detected in the IFF detection, some may be not, and that might be the reason that some antibodies are fit for IFF detection in PET tissues.

Another interesting finding in our study is that IHC could be continued to be performed after IFF test on the sections of PET tissues, while it hard to see IFF staining after IHC counterstaining were completed. The reason may due to the hematoxylin counterstaining during the IHC protocols. It was reported that hematoxylin counterstaining nearly completely abolish specific IF [7]. So we suggest that if you simultaneously do IIF and IHC staining, it is better to do IIF test first.

Collectively, in this study, we compare the detection rate of two mostly often used methods IHC and IFF in PET tissues and found that in some Abs, IIF method can be similarly applicable in the PET tissues as IHC method, while in other Abs, the IIF method is unbefitting in PET tissues. Our results also showed that IHC method can be executable after IIF method was finished in the same PET tissues.

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## Disclosure of conflict of interest

None.

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