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Original Article

On-Slide Clearing and Imaging of 100-µm-Thick Histological Sections Using Ethyl Cinnamate and Epifluorescence

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Abstract

Introduction: Thick histological samples are difficult to image without proper tissue clearing methods. Among these methods ethyl cinnamate (ECi)-based clearing preserves antigenicity and is compatible with immunofluorescent labeling. In contrast to many other clearing protocols, ECi-based clearing is fast and is done as a final step after standard immunofluorescent labeling protocols.

Aim: We aimed to develop a simple method of ECi-based tissue clearing approach of thick (100 µm) sections attached to a slide glass.

Materials and methods: Samples of human colorectal cancer underwent fixation and frozen section. We used indirect immunofluorescence to label Von-Willebrand factor, which is expressed in blood vessels. After completion of labeling and nuclear DAPI staining, the material was dehydrated using alcohols. Finally, the material was cleared and mounted with ECi and subsequently visualized via standard widefield epifluorescence microscopy. Image analysis of z-stacks were evaluated for the depth of visible signals and compared them to non-cleared samples.

Results: Compared to the non-cleared sections, the ECi-cleared sections provided a 2.5-fold increase in the observable tissue thickness following immunofluorescent staining. Further, the proposed approach is time efficient (4 days from tissue preparation to final imaging) as compared to other tissue clearing methods and low cost as it minimizes the use of large amounts of reagents.

Conclusion: ECi-based clearing is a very effective simple extension of standard immunofluorescent protocols and can be implemented in future experiments.

Keywords

colorectal cancer, epifluorescence, tissue clearing, ethyl cinnamate

INTRODUCTION

The inhomogeneity of light scattered by the tissue is a fundamental problem that leads to undesirable effects when histological tissue samples are observed.[1] The light emitted from the microscope slows down as it passes through the sample, which is due to cellular constituents such as lipids, carbohydrates and pigments which all have different refractive indices.[2] As a result, the light emitted by the sample is scattered in many directions, thus creating an opaque

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appearance of the tissue[3], particularly in thick sections. To counteract this problem, methods commonly known as tissue clearing have been introduced. Tissue clearing is now commonly used to match the refractive indices of a tissue sample to the refractive index of the clearing medium.[4] This is achieved by removing mostly the lipids from the cell with chemical compounds and replacing the lipids with the clearing medium, while preserving tissue integrity, and in some protocols also the antigenicity of the tissue. The latter is a major factor when the experiments require immunostainings.[5] There are a variety of ways to clear a tissue. As we have previously summarized, there are non-aqueous/solvent-based clearing approaches and aqueous-based clearing techniques.[6] Solvent-based clearing concentrates on dehydrating a tissue, followed by incubating it in the clearing medium, which matches the refractive indices of the surrounding medium and the tissue; macroscopically, the tissue becomes (nearly) transparent.[7] However, lipids and water are lost during the dehydration process which might restrict the useability of immunofluorescent staining. Furthermore, tissue shrinkage can be observed, and the toxicity of organic solvents can be problematic. Another approach is the aqueous-based clearing. The main techniques here are either simple immersion or hyperhydration, respectively.[1] Simple immersion matches the refractive index of the tissue by incubating it in a high-refractive index aqueous solution, such as formamide. It does not remove lipids, and thus the slight milky appearance of the tissue is preserved. Hyperhydration focuses on lowering the refractive index of the tissue itself by removing lipids. For instance, the CUBIC method[8] utilizes high levels of basic amino alcohols to effectively remove lipids, but as a possible problem, a change in the morphology can also be observed when the tissue sample gains size.[8]

In this study, we focus on the application of a non-aqueous-based clearing method which utilizes ethyl cinnamate (ECi) as the main clearing agent. Ethyl cinnamate is a low-toxic, high-refractive index liquid providing optimal transparency even after short incubation periods. It allows the preservation of antigenicity and offers exceptional compatibility with immunofluorescent labeling. The method was first introduced by W. Masselink et. al.[7] and used for clearing human organoids, *Drosophila melanogaster*, zebrafish, axolotl, and *Xenopus laevis* in the form of larger pieces of tissue.

Our approach focuses on clearing slide glass-attached histological specimens of up to 300 µm in thickness, i.e., significantly thicker than the 10–25 µm thick frozen sections typically used for immunostaining experiments. Imaging of blood vessels and nerve fibers in 3D might be improved when larger proportions of these structures are captured within a single section. Ethyl cinnamate-based clearing is a simple and quick approach when compared to other tissue clearing methods, taking only about 4 days to fully prepare the tissue sample for imaging versus 10 or more days for other approaches.

As a proof of principle, in our study we used human colorectal cancer tissue, which, due to its dense structure, is challenging as a target of tissue clearing.

AIM

To demonstrate a simple and fast on-slide clearing of thick tissue sections using ethyl cinnamate, preserving signals of immunofluorescent labeling and tissue morphology.

MATERIALS AND METHODS

Sample acquisition

Samples of human colorectal adenocarcinomas were acquired at St. Marina University Hospital, Varna. Surgeries were done open or minimally invasive robotic-assisted laparotomy using the Da Vinci Xi System (Intuitive Surgical). Tissue samples were collected following permission from the Ethics Committee of Prof. Dr. Paraskev Stoyanov Medical University in Varna (No. 39/07.07.2014). Fresh surgical material was immediately fixed, brought to the pathology department, and thoroughly examined **(Table 1)**.

Tissue processing

Tissue samples were processed by cutting 10×10×5 mm slices. These slices were cryoprotected in sucrose solution and subsequently submerged in Tissue-Tek O.C.T. for 5 min and standard blocks for frozen section were prepared. Sectioning was done at 100 µm, 200 µm, and 300 µm thickness on a cryostat. Sections were kept on Superfrost microscopic slides (Thermo Scientific) and dried for 12 hours at 37°C to achieve proper adhesion to the glass surface (bonding). The tissue was then rehydrated in PBS for 2 hours, blocking

Table 1. Data of cases of colorectal cancer patients used in this study

No	Gender	Tumor location	Histological type	Grade	T	N	M	Necrosis	Stromal reaction	Operative procedure
1	M	Rectum	Adenocarcinoma	G2	2	x	x	Yes	Yes	Robot assisted
2	F	Rectum	Adenocarcinoma	G2	2	1	x	No	No	Robot assisted
3	M	Sigma	Adenocarcinoma	G2	3	x	x	No	Yes	Laparotomia mediana
4	F	Rectum	Adenocarcinoma	G2	3	0	x	No	Yes	Laparotomia mediana

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was done for 8 hours using antibody diluent (100 mg Gelatine, 50 mg NaN_3, 0.5 ml Triton X-100 in 100 ml PBS) before we applied primary antibodies against von Willebrand factor (DAKO, vWF polyclonal rabbit anti-human antibody, A0082) for 24 hours at room temperature (dilution 1:100 in antibody diluent). The tissue was then thoroughly washed in PBS, and we incubated a mixture of the secondary antibody (Invitrogen Alexa Fluor 488 goat anti-rabbit IgG (H+L), A11034, diluted 1:200) and DAPI (Invitrogen D1306, 5 µg/ml). We then post-fixed the tissue using a 4% formaldehyde solution for 1 hour at room temperature. After another washing step in PBS we dehydrated the tissue in an increasing ethyl-alcohol (EtOH) chain ending in pure isopropyl alcohol.

The clearing started by application of several drops of ECi to fully cover the tissue on the slide. We incubated it for 10 minutes and replaced the ECi by blotting off the excess liquid and reapplying ECi to cover the tissue. After the second incubation of 10 minutes, we again replaced the ECi as described and applied a smaller amount of ECi (estimated to fill the gap between the glass slide and the cover slip) and coverslipped the tissue without any pressure. All steps are listed in **Table 2.**

Image acquisition and processing

Image acquisition of the samples was done with an AxioImager Z.2 (Zeiss, Germany) using standard epifluorescence with a 20× magnification objective (EC Plan-NEOFLUAR, NA=0.5). We acquired z-stacks of different regions covering the whole tissue thickness. We chose the optimal z-step value fulfilling the Nyquist criterion giving the final voxel size of 0.322×0.322×1.23 µm. The same settings were used for the cleared and non-cleared (control) samples. Image data was exported in the TIFF format and loaded in Fiji[9] for 3D visualization. We measured (n=15) the depth of visible and "usable" signals along the z-axis with equal settings for both images of control and cleared tissue and performed a two-tailed t-test to confirm statistically significant increase in transparency.

RESULTS

Within minutes of incubation in ECi, the tissue section's macroscopic appearance changed from white and opaque to transparent **(Fig. 1)**. The second ECi incubation made the tissue nearly fully transparent **(Fig. 1)**.

Moreover, we were able to show that the visible depth into the tissue samples using standard epifluorescence microscopy was significantly (p<0.001) increased compared to a non-cleared tissue sample **(Fig. 2)**. Non-cleared tissue samples had a detectable immunofluorescent signal up to a depth of 32.7±1.98 µm along the z-axis, while in the cleared tissue we were able to measure signals up to 81.1±16.30 µm of depth. This equals approximately a 2.5-fold increase in

Table 2. Detailed clearing protocol with immunofluorescent labeling steps

	Step	Description	Timing
1	Cryosection	100 µm – 300 µm in O.C.T.	–21°C
2	Bonding		12 h, 37°C
3	Rehydration	PBS	2 h, room temp.
4	Blocking	Antibody diluent	8 h, room temp.
5	Primary antibody	In antibody diluent	24 h, room temp.
6	Washing	PBS	3×2 h, room temp.
7	Secondary antibody + DAPI	In PBS	24 h, room temp.
8	Washing	PBS	3×2 h, room temp.
9	Post-fixation	4% formaldehyde	1 h, room temp.
10	Washing	PBS	3×2 h, room temp.
11	Dehydration	50% EtOH	20 min
		70% EtOH	20 min
		90% EtOH	10 min
		96% EtOH	10 min
		100% EtOH	2×10 min
		100% isopropyl alcohol	2×10 min
12	Clearing	1. ECi	10 min
		2. ECi	10 min
13	Mounting	ECi	
14	Imaging		

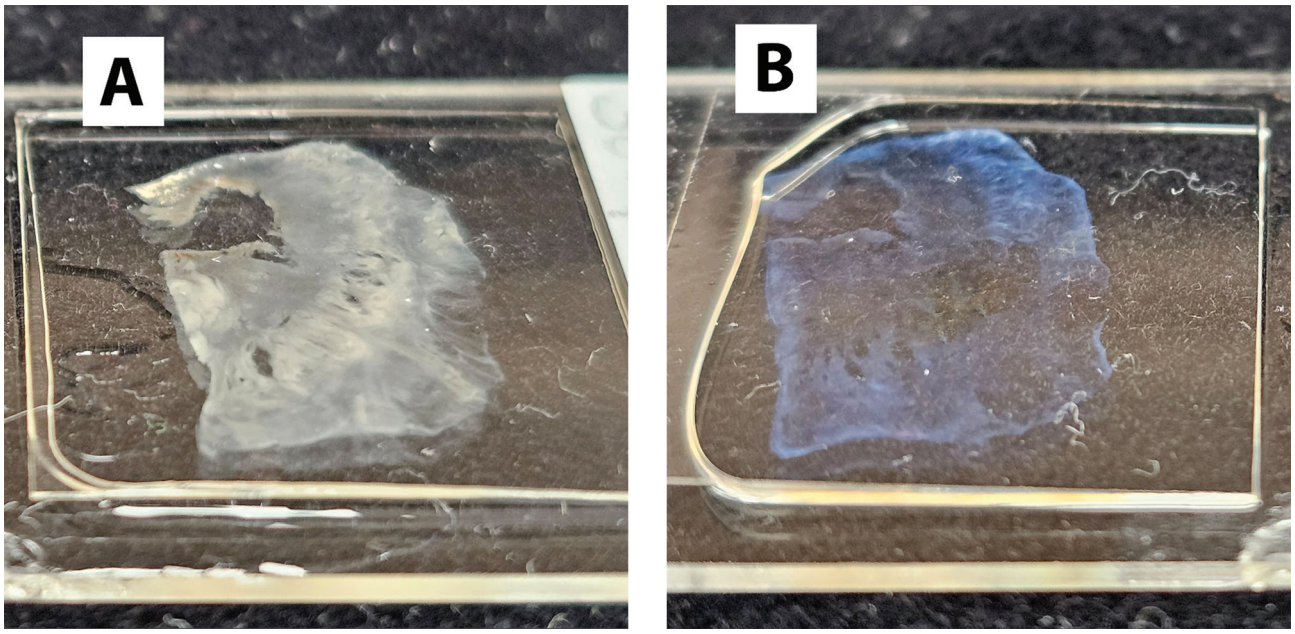

Figure 1. Macroscopic visualization of cleared and non-cleared specimens. Macroscopic appearance of 300 μm thick sections of human colorectal cancer in traditional non-cleared (**A**) versus cleared (**B**) mounted sections.

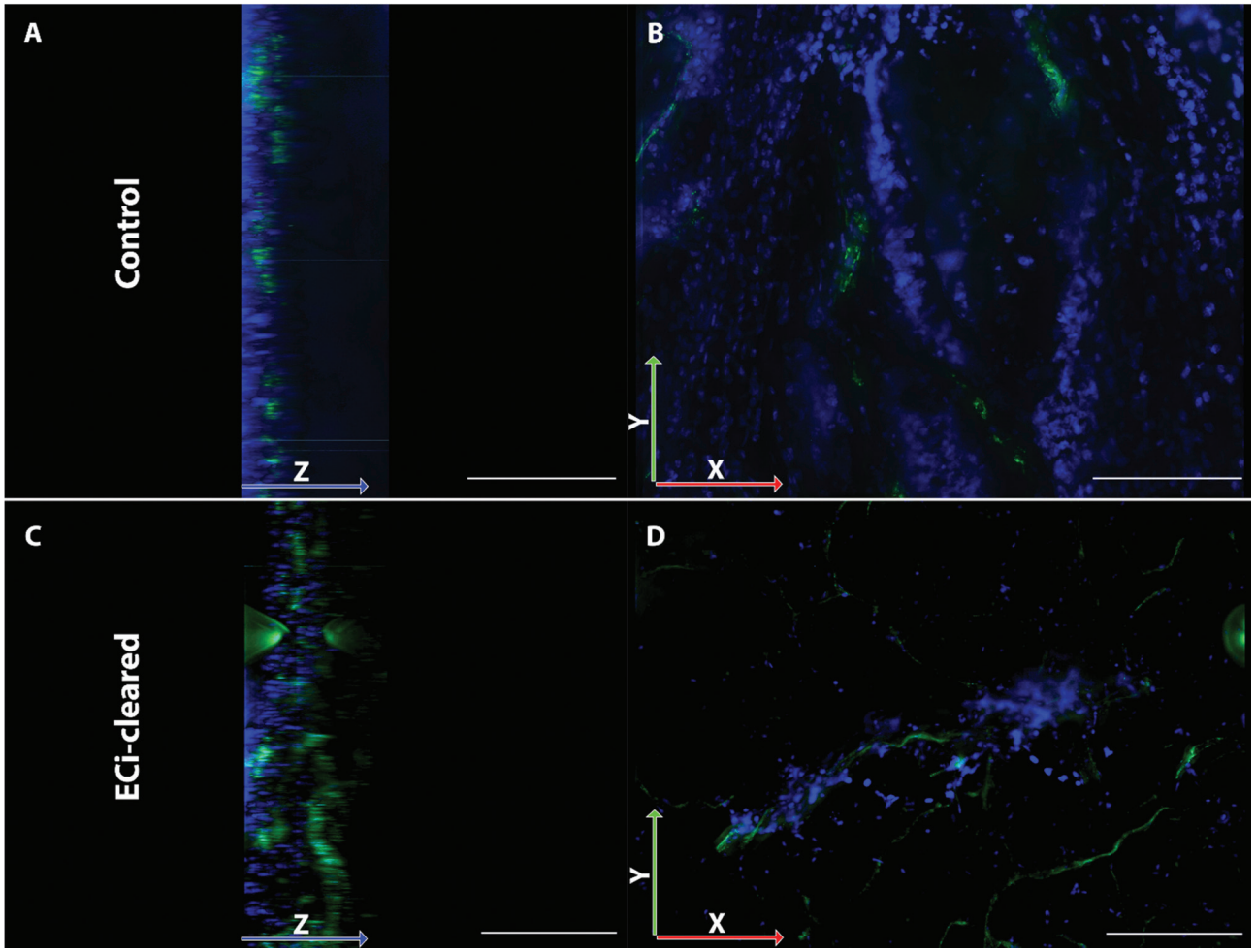

Figure 2. Microscopic visualization of cleared and non-cleared specimens; 3D representation of microscopic images of non-cleared (**A, B**) or cleared (**C, D**) samples in two orthogonal projections. It can be seen that the detectable signal in non-cleared tissue (**A**) reaches a depth of approximately 33 μm, while the signal in cleared tissue (**C**) can be easily detected at a depth of more than 80 μm from the surface (in the orthogonal projections **A** and **C** the upper surface is here on the left). Scale bar 100 μm.

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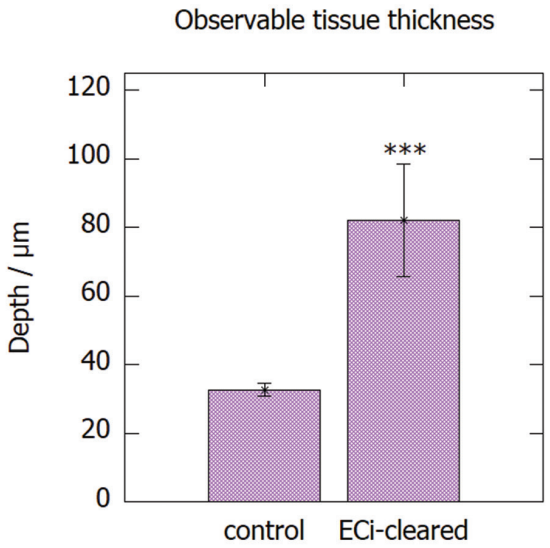

Figure 3. Observable tissue thickness. Statistically significant increase (p<0.001) in the observable tissue thickness after clearing showing an ~2.5-fold increase.

observable tissue thickness **(Fig. 3)**.

The tissue integrity was well preserved. We did not observe macroscopic tissue shrinkage or expansion and delicate structures, for example blood vessels were clearly identifiable.

DISCUSSION

We propose our protocol for investigation of tissue samples in laboratories with available standard epifluorescence microscopy. Additional workload and effort may be further reduced, if larger quantities of ECi are available and the microscopic slides could be dipped (instead of repetitive application of drops onto the slide). ECi-on-slide-clearing is a straightforward, cheap and effective way to improve imaging.

We chose samples of colorectal cancer, because this type of tissue is very opaque, and clearing is critical if images of more than 40 µm thickness need to be acquired. We were able to show that clearing tissue sections on microscopic slides with ECi as the main reagent is achievable. In fact, the ECi incubation times in the range of minutes rendered the samples macroscopically transparent. Our clearing protocol is compatible with immunofluorescent labeling and results in a significant increase in observable tissue thickness when compared to non-cleared samples.

Further improvement is to be expected if the proposed clearing approach is combined with more advanced imaging modalities such as confocal or light-sheet microscopy.

CONCLUSION

We have shown that On-Slide-ECi-clearing is a simple and effective extension for standard immunofluorescent protocols. Future experiments setups targeting specific research questions on different tissues such as colorectal cancer will benefit from the ECi-clearing protocol.

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Competing Interests

The authors have declared that no competing interests exist.

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Очистка и визуализация на предметном стекле гистологических срезов толщиной 100 μm с использованием этилциннамата и эпифлуоресценции

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Резюме

Введение: Плотные гистологические образцы трудно визуализировать без надлежащих методов очистки тканей. Среди этих методов очистка на основе этилциннамата (ECi) сохраняет антигенность и совместима с иммунофлуоресцентной маркировкой. В отличие от многих других протоколов очистки, очистка на основе ECi быстрая и выполняется в качестве последнего шага после стандартных протоколов иммунофлуоресцентной маркировки.

Цель: Мы стремились разработать простой метод очистки тканей на основе ECi плотных (100 μm) срезов, прикреплённых к предметному стеклу.

Материалы и методы: Образцы колоректального рака человека подвергались фиксации и замораживанию срезов. Мы использовали непрямую иммунофлуоресценцию для маркировки фактора Von-Willebrand, который экспрессируется в кровеносных сосудах.

После завершения маркировки и ядерного окрашивания DAPI материал был дегидратирован с использованием спиртов. Наконец, материал был очищен и закреплён с помощью ECi, а затем визуализирован с помощью стандартной широкопольной эпифлуоресцентной микроскопии. Анализ изображений z-стеков мы оценили на предмет глубины видимых сигналов и сравнили их с неочищенными образцами.

Результаты: По сравнению с неочищенными срезами очищенные ECi срезы обеспечили 2,5-кратное увеличение наблюдаемой толщины ткани после иммунофлуоресцентного окрашивания. Кроме того, предлагаемый подход является эффективным по времени (4 дня от подготовки ткани до окончательной визуализации) по сравнению с другими методами очистки тканей и недорогим, поскольку он сводит к минимуму использование большого количества реагентов.

Вывод: Очистка на основе ECi является очень эффективным простым расширением стандартных иммунофлуоресцентных протоколов и может быть реализована в будущих экспериментах.

Ключевые слова

колоректальный рак, эпифлуоресценция, очищение тканей, этилциннамат