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Article

Fast Green FCF Improves Depiction of Extracellular Matrix in Ex Vivo Fluorescence Confocal Microscopy

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Abstract: Rapid microscopic analysis of tissue is an essential diagnostic tool in oncological surgery. The gold standard for intraoperative histological tissue evaluation are frozen sections. However, frozen sections are prone to a variety of artefacts and require skilled staff and specialized lab equipment. A potential method for rapid intraoperative tissue evaluation that does not require fixation, freezing nor sectioning of the tissue, is ex vivo fluorescence confocal microscopy (FCM). The visualization of the structurally important extracellular matrix (ECM) in conventional ex vivo FCM lags behind the standards of conventional histology. The objective of this study was to find a stain that will improve the depiction of the ECM to resemble FFPE H&E sections as closely as possible.11 different tissue stains were tested on 122 tissue samples submitted to the Department of Pathology at the Medical University of Vienna. The study was conducted on the RS-G4 Upright (Caliber I.D. Rochester, NY, USA, distributed in Europe by MAVIG GmbH, Munich, Germany).Fast Green FCF (FGFCF) in combination with Acridine Orange as nuclear stain improved the visibility of structural details of ECM. Morphological details in FCM were equivalent or even superior to frozen sections in most analyzed categories. The addition of FGFCF to the conventional staining protocol improves the assessment of ECM or analysis of fibrosis. The rapid staining protocol is compatible with an application in intraoperative microcopy.

Keywords: digital pathology; ex vivo fluorescence confocal microscopy; extracellular matrix; frozen sections

1. Introduction

Rapid microscopic tissue analysis is a pillar of pathology and has been used for decades in the form of frozen sections for quick intraoperative diagnosis of malignancy and the assessment of resection margins in tumor surgery. Frozen sections do, however, have marked disadvantages, such as a low quality due to freezing artefacts, especially in tissues with little tissue cohesion or lipid-rich tissues, and the need for extensive lab equipment and specially trained personnel as well as the loss of the tissue for further investigations [1].

Fluorescence confocal microscopy (FCM) has, in the past few years, emerged as a relevant tool for microscopic tissue analysis that may serve as an ancillary method in rapid tissue diagnostics or potentially even replace them [2–5]. FCM is a fast virtual microscopy technology that produces digital microscopic images from tissue samples without prior fixation, freezing or sectioning.

In a previous study we have investigated whether FCM is a suitable tool for rapid tissue analysis of specimen rich in adipose tissue, such as breast tissue [6].

We found that FCM can be useful in the analysis of these specimen and the depiction of cellular nuclei was excellent and comparable to hematoxylin and eosin (H&E) stained slides. However, we also noticed that extracellular matrix (ECM) was generally less well defined than in conventional histology and showed a different texture.

ECM is an integral part of all tissues and consists of various proteins with an often fibrillary structure and functions as a stabilizing framework [7]. The amount and the histologically visible texture of ECM is tissue-specific but highly variable among different areas of the body. Moreover,

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pathologic conditions like inflammation can trigger excess deposition of ECM, a process that is called fibrosis. It may occur in almost all types of tissues and frequently leads to impairment of organ function. Specific structures of normal ECM are the basement membranes (BM) that delineate epithelial cell compartments in many tissue types like the skin and various glandular organs. Beyond its functional role in defining tissue architecture and guiding cell growth it is also of outstanding diagnostic importance in oncology. Many treatment choices depend on correctly identifying the level of invasiveness of the tumor. The basement membrane and the rest of the ECM should therefore be depicted in as much detail as possible. It should match the current gold-standard for ECM evaluation, namely H&E-stained histology sections, as closely as possible.

In this follow-up study we investigated potential staining options for a better depiction of ECM, that can both 1) be implemented in FCM and 2) be performed in a sufficiently short amount of time to be suitable for the application of FCM in rapid tissue assessment.

2. Materials and Methods

Patients

This study was approved by the local Ethics Committee (protocol number: 1872/2019). 122 tissue samples from 18 patients were included. Samples were anonymized without possibility of tracing them back to the respective patient.

Tissue Specimen

7 different tissue types were included in the study:
nontumor specimen: skin, liver, colon, kidney, greater omentum and
tumor specimen: mesothelioma and liver metastasis from colon cancer.
Samples were up to 1cm in length and width and 1-3 mm in height.

After harvesting, samples were immediately frozen and stored at -20C. Thawing tissue was performed by wrapping it in saline-soaked gauze.

Instrument

The study was conducted on the RS-G4 Upright (Caliber I.D. Rochester, NY, USA, distributed in Europe by MAVIG GmbH, Munich, Germany).

RS-G4 is an upright research confocal microscope built to scan large-scale mosaics up to 120 x 80 mm with a maximum resolution of 1024x1024 pixels at 5.9 frames per second. The operating wavelengths are 405nm, 488nm, 561nm, 640nm (all for fluorescence) and 785nm (reflectance). All available wavelengths were employed except for 405nm.

Reagents and Staining Protocol

7 dyes were tested for their ability to depict ECM in a superior fashion, compared to the instrument's standard set-up, which employs the fluorescent dye Acridine Orange for staining nuclei and the so-called reflectance, which is based on the amount of light that is reflected by a surface, for visualizing unstained tissue components like ECM or cytoplasm of cells.

All potentially suitable dyes (COL-F, Direct Red 80, Fast Green FCF, Methyl Blue, WGA, PAS and Eosin) were chosen based on promising results mentioned in literature or their proven ability to stain ECM in conventional histology as well as their fluorescence features with excitation and emission wavelengths compatible to the microscope lasers. Specifically, we chose PAS and WGA for their expected ability to highlight basement membranes as well as eosin, which is the standard stain in pathology that pathologists are used to. The other stains were chosen based on literature reports.

All samples were incubated in the respective dye (with or without previous immersion in 96% ethanol) and then rinsed in PBS for 15 seconds (Figure 1).

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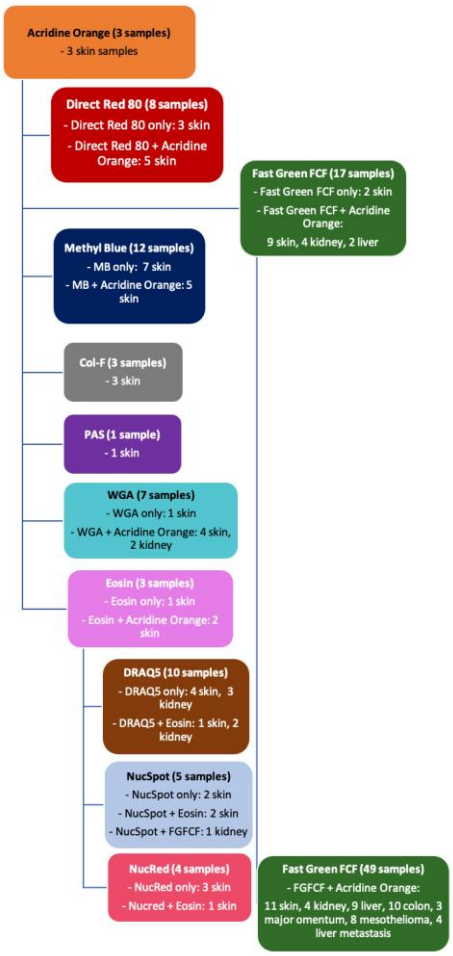

Figure 1. Flowchart of staining process: Order of stains and amount and types of tissue samples which were stained.

Staining and Processing

For staining tissue samples, plastic molds (Tissue-Tek® Cryomold® from Sakura Finetek, Staufen im Breisgau, Germany) were used in 15x15x5 mm (1,125 ml) and 25x20x5 mm (2,5 ml). The plastic molds were filled with the required stain or reagent and the tissue sample was immersed into the mold by forceps. During the staining, the tissue was lightly stirred in the mold with the forceps.

After testing, samples were processed according to in-house protocol for regular FFPE specimens. For comparison with frozen section histology, HE-stained frozen sections were cut from the samples processed for FCM and additionally from similar but unrelated tissue specimen that were entirely untreated.

Image Evaluation

For evaluation and comparison all physical slides were scanned (PANORAMIC 250 Flash III, 3D Histech) and all images acquired with the RSG4 were transferred to SlideViewer 2.5 (3DHISTECH Ltd., H- 1141 Budapest, Oev u. 3., Hungary).

The images were separately analyzed by two pathologists. The analysis was performed based on the following parameters:

(1) Nuclear morphology
(2) Cell borders
(3) Texture of extracellular matrix
(4) Demarcation of parenchyma and stroma
(5) Structural integrity

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(6) Perceptibility of key structures (blood vessels, connective tissue septa or borders of compartments)
(7) Borders of the tissue specimen

Each parameter was separately scored by two pathologists on a scale from 1 (inadequate) to 5 (comparable to traditional FFPE H&E sections).

Whole slide images from frozen sections were included as well to compare our findings to current standard of rapid histologic tissue analysis. For frozen section comparison we analyzed 3 skin samples, 3 kidney samples, 2 liver samples, 3 adipose tissue samples, one colon sample and one adenocarcinoma of the colon sample.

Statistics

Sample characteristics were reported on by applying the appropriate method according to the type of value: mean (standard deviation, SD), median (Interquartile ranges, IQR) or absolute numbers. All statistical analyses were performed using the most recent version at the time of analysis of SPSS (IBM SPSS® Statistics 27) and R (R 4.2.3 for Mac).

3. Results

3.1. Evaluation of ECM Stains

COL-F, Direct Red 80, Eosin, Fast Green FCF, Methyl Blue, PAS and WGA were all considered potential candidates for ECM staining based on the criteria mentioned above. During initial testing, Eosin showed the most promising results with regards to staining all parts of the ECM in an even manner, with the additional benefit of looking familiar to the pathologist's eye. It unfortunately proved to be entirely incompatible with the nuclear stain acridine orange, which was by far superior to all other nuclear stains tested and therefore was the only choice for staining nuclei. Other stains showed inhomogeneous staining patterns, e.g. PAS and WGA, and were excluded from further testing (for an overview of all evaluations see Table 1).

The above-mentioned evaluation and selection of stains resulted in the following protocol: staining the tissue sample for 90 seconds in Fast Green FCF (1:30 dilution in PBS), rinsing it in PBS, then staining it with Acridine Orange (1:4 dilution with PBS) for 1 minute and eventually rinsing it in PBS.

Table 1. Summary of the evaluation of all stains tested.

ECM STAINS.	STAINING RESULT	FINAL SELECTION	REASON FOR SELECTION
DIRECT RED 80	Decent ECM staining	×	Overlaying nuclear stain, inhomogeneities
FAST GREEN FCF	Satisfying ECM staining	✓	Detailed ECM staining, short staining time, compatible with acridine orange
METHYL BLUE	ECM staining, but less defined than *Fast Green FCF*	×	Varying staining intensity, smearing
COL-F	Decent ECM staining	×	Only excitable on the same wavelength as Acridine Orange
PAS	ECM staining, partially basal lamina staining	×	Irregular staining pattern
WGA	ECM staining, partial basal lamina staining	×	Inhomogenous staining pattern

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EOSIN	Satisfying ECM staining	×	Not compatible with Acridine Orange
NUCLEAR STAINS			
ACRIDINE ORANGE	Satisfactory nuclear depiction	✓	Decent nuclear detail, short staining time
DRAQ5	Nuclei were stained, but only using high laser power and PMT	×	Blurred nuclei in pseudocolor mode
NUCSPOT	Decent nuclear depiction	×	Not compatible in combination with Eosin
NUCRED	Nuclei were stained, but only using high laser power and PMT	×	Blurred nuclei in pseudocolor mode

3.2. Overall Representation of ECM Texture in Connective Tissue with Fast Green FCF

The fibrillar structure of ECM, which appeared cloudy and speckled in Acridine Orange + Reflectance was well defined with the addition of Fast Green FCF (Fig 2). Higher magnification confirmed this impression. As the ECM structures presented better-defined, improving the visibility of the borders between cells and ECM were better identifiable in comparison to Acridine Orange + Reflectance. The overall ECM staining in Acridine Orange + Fast Green FCF seemed more homogenous and less blotty as it did in Acridine Orange + Reflectance.

Another noticeable difference was the more intense visualization of elastic fibers in addition to collagen bundles in Acridine Orange + Fast Green FCF as both stains, especially Fast Green FCF, stained them (Figure 2). The depiction of nuclei was unaffected by Fast Green FCF.

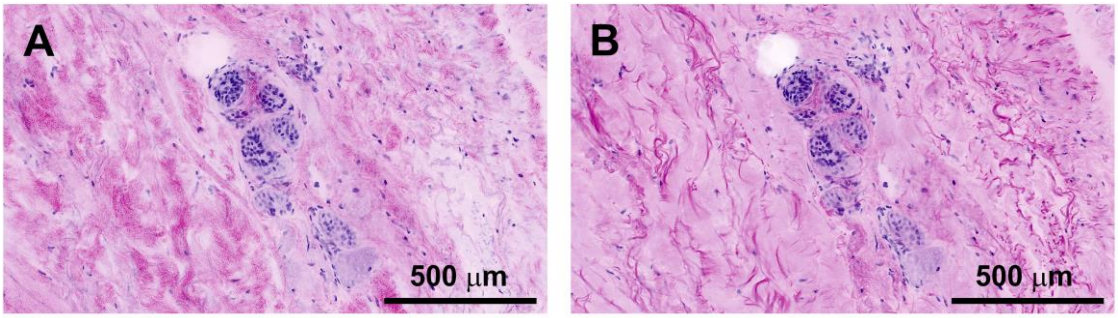

Figure 2. Comparison of FCM: Acridine Orange + Reflectance (A) and CM: Acridine Orange + Fast Green FCF (B) in sweat glands within the dermis of the skin.

Following this selection, FCM employing Fast Green FCF was systematically compared to FCM without addition of Fast Green FCF based on the 7 tissue parameters described above in an attempt to quantify the differences (Figure 3).

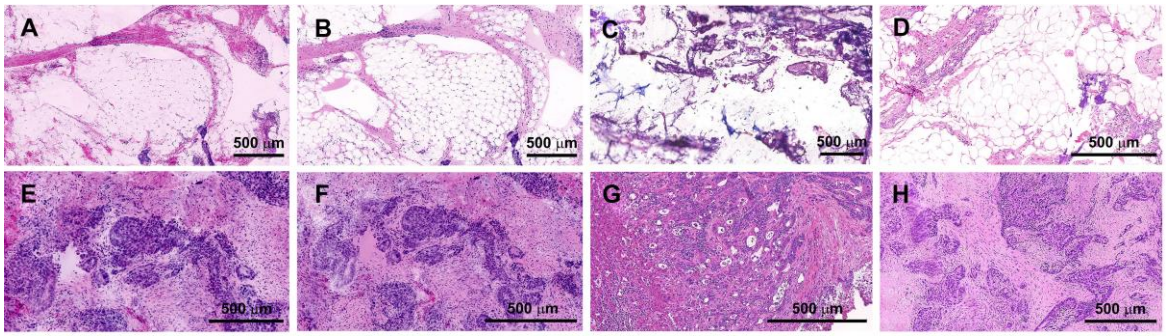

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Figure 3. Comparison of FCM: Acridine Orange + Reflectance (A,E), CM: Acridine Orange + Fast Green FCF (B, F), Frozen section (C, G) and FFPE (D, H), HE.

A,B, C, D major omentum, E, F, G, H mesothelioma

3.3. Display of Cellular Details and Other Tissue Structures

In an attempt to quantify differences between the two staining procedures we scored seven key morphologic parameters (nuclear morphology, cell borders, texture of ECM, demarcation of parenchyma and stroma, structural integrity, perceptibility of key structures, borders of the tissue specimen) as described above.

Notably, AO+FGFCF received higher scores than AO+Reflectance in most/all tissue samples in the categories "texture of ECM", "demarcation of parenchyma and stroma" as well as "perceptibility of key structures". The structural integrity and visibility of the borders of the tissue specimen were equivalent in both procedures, while the demarcation of cell borders showed better scoring for FGFCF only in adipose tissue and the colon sample.

When comparing FCM to frozen sectioning, both AO+FGFCF and AO+Reflectance received consistently higher scores for all parameters in adipose tissue.

For the other types of tissues we found that FCM performed better in the categories of structural integrity (except for kidney), perceptibility of key features (except liver) and tissue borders (except kidney). Frozen section on the other hand performed better for the parameters demarcation of parenchyma (except liver) and depiction of cell borders.

Both FCM and frozen section performed mostly equivalent for nuclear morphology, frozen section did however perform worse in liver, liver metastasis and colon samples.

The morphologic detail of extracellular matrix was best represented by AO+FGFCF in adipose tissue, skin, liver and mesothelioma, while it was roughly equivalent in the other tissues.

3.4. Total Processing Time for CM Was Generally Short

The processing time for tissue samples stained with Acridine Orange + Fast Green FCF was approximately 2 minutes for staining and approximately 1 minute for accurate slide covering and positioning onto the stage holder of the microscope. Scanning and post processing time depended on the size of the tissue sample, between 5 and 20 minutes (in rare cases).

4. Discussion

The major strength of ex vivo FCM is the ability to perform nondestructive microscopic assessments of tissues without prior fixation or other kinds of conventional histologic workup. The whole procedure can be completed within minutes, a time frame comparable to conventional frozen section diagnostics. In addition, FCM avoids tissue damage associated with freezing and is performed without loss of tissue by sectioning. It also does not require a specially equipped histology laboratory with adequately trained staff.

There are several areas where ex vivo FCM might be a valuable addition or even alternative to (frozen section) histology: the non-destructive microscopic investigation of small tissue samples in both research and diagnostics, which includes potential assessment of tissue adequacy prior to further workup with conventional methods. Or as an option for intraoperative diagnostic histology at institutions that do not have frozen section histology services available on-site.

For these reasons, ex vivo CM is currently being studied intensely, and is already applied in some fields, such as dermatology or urology, as a feasible and fast method for tissue evaluation without the need of fixation [2–5,8–12].

By using Acridine Orange as a nuclear stain and the reflectance laser for depiction of other tissue components, a realistic presentation of different tissue types can be created digitally within a short time frame. The images are being digitally coloured in order to achieve an appearance similar to H&E- stained histology slides thereby rendering them ideal for pathologists.

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However, despite the familiar looking staining pattern, we found in a previous study that the visualization of ECM by reflectance laser was unsatisfactory when examined in greater detail [6]. Although the morphology of nuclei was excellent in FCM, the relationship of cells to their surroundings was not always well defined. In particular, it was often difficult to identify the type and structure of connective tissue as staining intensity in reflectance does not necessarily reflect tissue density, as would be the case in conventionally stained tissue sections. The pattern and distribution of staining signals in reflectance also differ from histologic sections as they often do not match the texture of connective tissue in H&E staining. Especially when considering implementation of this novel technique to cancer diagnostics, e.g. the evaluation of the invasiveness of a tumor, a reliable representation of ECM is crucial.

The dyes for ECM included in this study were selected on the basis of producing a known and at least decent ECM stain in either conventional histology or fluorescent microscopy. During testing of the different stains, we had to exclude most of the ECM stains for a variety of reasons such as staining inhomogeneities and an irregular staining pattern, as was the case for Direct Red 80, Methyl Blue, PAS and WGA. Col-F showed a decent ECM staining but required excitation at 488nm, the same wavelength needed for excitation of Acridine Orange.

Our selection process revealed that a combination of Acridine Orange and Fast Green FCF yielded the best overall results, with improved ECM depiction in several organ samples and a depiction of structures comparable to HE stained histology sections.

Given the fact that Acridine Orange + Fast Green FCF outperformed Acridine Orange + Reflectance in almost every parameter (most markedly in “presentation of the extracellular matrix” and particularly in adipose tissue), this staining combination holds potential both for research and clinical application:

The better depiction of extracellular matrix might be of value in every application where the structure and amount of fibrotic tissue is of interest, e.g. fibrosis/desmoplasia in the context of a tumor or in chronic inflammation.

Other features of this combination were a short staining time (ca. 2 minutes) and a simple staining protocol, which would make it a feasible method for rapid tissue analysis. We therefore compared FCM to frozen sections. In terms of morphologic detail, FCM was indeed equivalent or even clearly superior (adipose tissue) except for visibility of cell borders and the demarcation of parenchyma and stroma.

A notable point in the design of this study was the use of the RS-G4 microscope, which, while ideal for identifying novel staining combinations, is mainly designed for research purposes and not for clinical diagnostics. With its additional functions and modalities (such as a variety of lasers and laser settings), the duration of image processing was longer than it would usually be on a device intended for clinical use.

Another limitation of this study is the amount and somewhat limited selection of tissue samples, as the study was conducted on left-over tissue from routine pathology that was not needed for further analysis.

Of note is also the fact, that an important target structure that could not be specifically highlighted by our method are the basement membranes. This fact might be of relevance for the detection of early phases of tumor invasion.

5. Conclusions

This study shows that by using the ECM stain Fast Green FCF in combination with the nuclear stain Acridine Orange, the histologic features of different tissue types can be displayed satisfactorily and, depending on tissue type, in a superior fashion to Acridine Orange + Reflectance by CM.

Our proposed method improves not only the depiction of the ECM in comparison to Acridine Orange + Reflectance but also the overall representation of tissue structures; especially in adipose tissue the improvements are of note. The protocol we developed is fast and easy to perform.

The total processing time is short enough for a potential diagnostic application, especially in the setting of intraoperative microscopy.

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Informed Consent Statement: Not applicable.

Data Availability Statement: Data will be made available by the corresponding author upon reasonable request.

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Conflicts of Interest: All authors declare that they have no financial or personal relationships that could be viewed as a potential conflict of interest.

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