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Original Research

Stented Vessels: A Challenge for Histological Preparation and Microscopy

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Abstract

Objective: The first procedure to treat blocked coronary arteries was coronary artery bypass graft surgery. In 1977, Andreas Grüntzig introduced percutaneous transluminal coronary angioplasty (PTCA). Today, several stent systems exist ranging from bare metal stents (BMS) to various drug-eluting stents. Unfortunately, our understanding of the arterial reaction to stent implantation is incomplete – primarily due to technical limitations in the histological study of stented vascular tissue.

Methods: In our study, we examined different histological preparation methods based on the embedding material methacrylate. The procedure of embedding and sectioning of stented porcine arteries was optimized for the specific requirements, like histochemistry, immunohistochemistry or pre-stained fluorescence. Furthermore, we used a microscopical technique described as fluorescence intensity decay shape analysis microscopy (FIDSAM) to eliminate auto-fluorescence from fluorescently labeled tissue.

Results: The sections were suitable for histochemical and immunohistochemical staining. Additionally, pre-labeled fluorescence in the porcine tissue was not lost by the embedding process.

Conclusions: The evaluation of arterial cross sections with FIDSAM technology gives new, very important insights into the examination possibilities of fluorescently labeled tissue. Future studies of the vascular response to a variety of new stent materials will provide important clues to the pathogenesis resulting in restenosis and occlusion of stents.

INTRODUCTION

One of the most common vascular diseases is atherosclerosis which is a leading cause of death in the western world [1]. The implementation of a procedure called percutaneous transluminal coronary angioplasty (PTCA) that can restore the obstruction or the narrowing of a blood vessel is one of the main treatments to cure atherosclerosis. In this procedure a stent which is mounted in a balloon catheter is inserted into the artery at the site of the initial blockage. Balloon angioplasty was introduced in 1977 when Andreas Gruentzig successfully achieved the first PTCA using a balloon catheter [2, 3]. However, PTCA can cause injury to the blood vessels leading to restenosis and particularly in-stent restenosis [4, 5] as occasionally 15-20% of the patients develop a re-narrowing after coronary stenting within six months [6]. Implantation of the stent leads to restenosis and is mainly characterized by excessive proliferation of neointimal cells. Mostly vascular smooth muscle cells (VSMCs) that migrate from the media into the intima, lead to re-narrowing of the artery [7, 8].

Unfortunately, our understanding of the arterial reaction to stent implantation is incomplete – primarily due to technical limitations in the histological study of stented vascular tissue [9]. Conventional paraffin embedding and sectioning procedures have well-known limitations in the histological study of vascular effects of endovascular stent implantation. In general, these procedures require the complete removal of the metal stent prior to tissue processing, thereby disrupting normal vascular architecture and, in particular, the

stent-tissue interface. To accurately observe the vascular response, it is imperative that the stent-tissue interface is sectioned with minimal damage to the tissue [10]. To this end, it is now common practice to employ methacrylate resins as the embedding media of choice [11, 12]. Methacrylate resins are successfully utilized as embedding media for hard biological tissues such as undecalcified bone samples, and these methods have previously been adapted or further modified to suit the study of stented vessels [13]. Subsequent sectioning can be carried out with tungsten carbide blades as well as with a saw and grinding tools [10].

Based on the desired examination, one should be aware that some embedding materials based on methacrylate are not suitable for fluorescently labeled tissue because their polymerization requires ultraviolet light. Additionally, some embedding materials are not suitable for immunohistochemistry because of high polymerization temperatures.

In this paper we describe in detail the embedding methods using different methacrylates and present a comparative review of their performance in terms of histological, histochemical, immunohistochemical and fluorescently labeled examinations. Figure 1 shows a brief overview of the different embedding and fixing materials that are suitable for the corresponding examination of stented vessels.

Additionally, we use a new approach to overcome high background auto-fluorescence in tissue, based on the recently described fluorescence intensity decay shape analysis microscopy (FIDSAM) [14]. The FIDSAM technology allows for the discrimination between the fluorescence signal of fluorescent labels and auto-fluorescent sample areas by analyzing their respective fluorescence lifetime decay patterns. Using FIDSAM we investigated the delivery of a reporter gene coding for green fluorescent protein (GFP), by which the stents were coated before implantation into ex vivo porcine arteries.

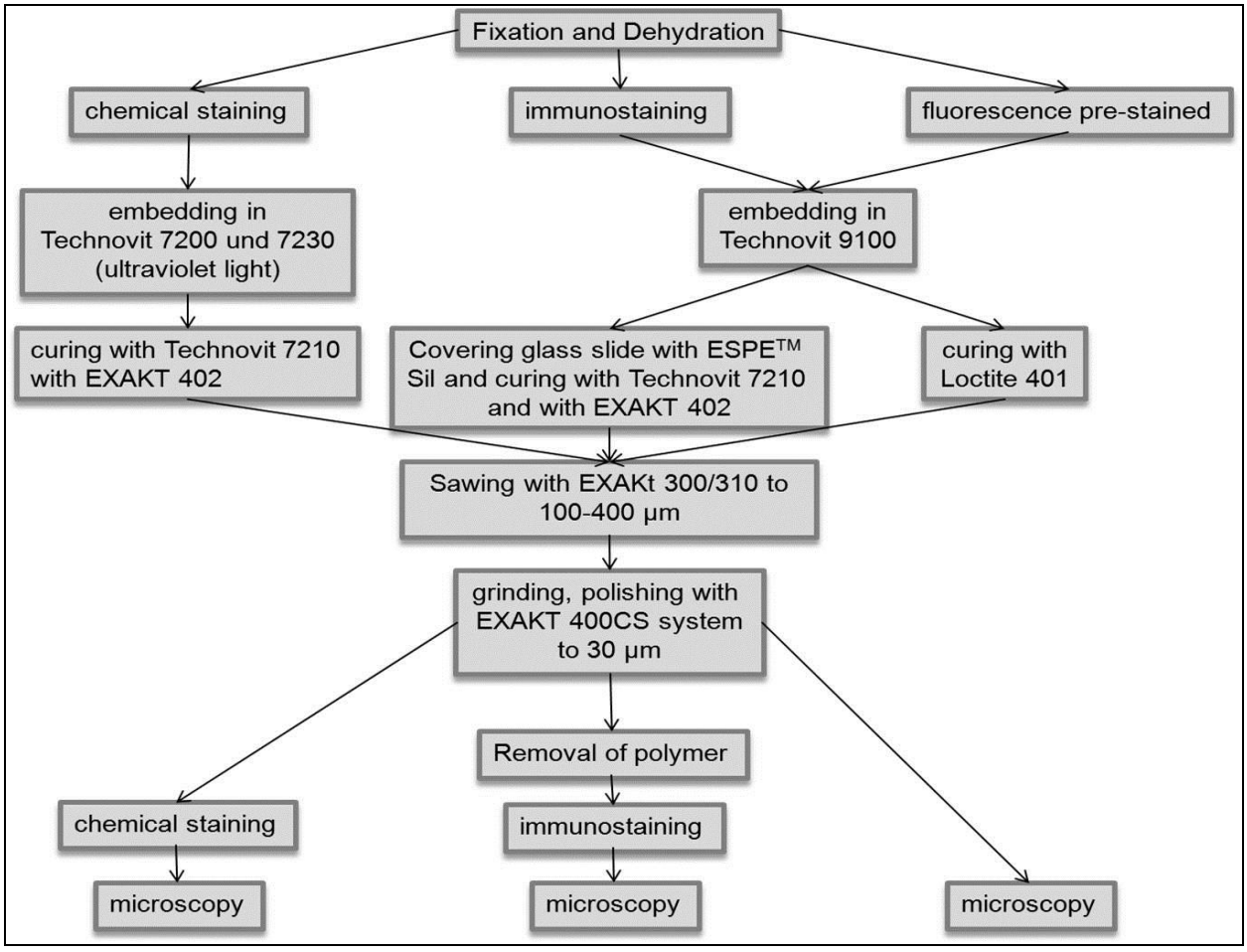

Fig. 1. Summary of the histological techniques to perform histochemistry, immunohistochemistry and pre-stained fluorescence imaging.

METHODS

Animals and Stents

The study was performed in accordance with the Federation of Laboratory Animal Science Associations (FELASA) and American Association for Laboratory Animal Science (AALAS) recommendations on the care and use of laboratory animals. Protocols and procedures were approved by the Animal Care and Welfare Commissioner of the University of Tuebingen. For histochemical stains, pig coronary arteries were stented (right coronary artery, left anterior descending artery or left coronary artery). The explantation of the stented arteries was conducted one month later. For immunohistochemistry, fluorophore and reporter gene-coated stents, carotid arteries from German landrace pigs of 45-70 kg were harvested and rinsed with PBS (phosphate buffered saline). Subsequently coronary BMS 4.5 x 20 mm from Qualimed (Winsen, Germany) were expanded in the vessels. After 24 h the vessels were washed with PBS and fixed.

Tissue fixation and dehydration

After explantation of the blood vessels they were rinsed with PBS and fixed in 4% PFA (paraformaldehyde) in PBS for 24 h. Thereafter, the vessels were stored in PBS until further preparation. For dehydration the fixed blood vessels were washed with running tap water and then dehydrated by ethanol and water in ascending ethanol concentrations of 50%, 70%, 90%, 96% and two times 100% for 30 min each.

Preparation for histochemistry

The embedding material for samples used to examine stented vessel by histochemistry was Technovit 7200. The fixed and dehydrated tissue was preinfiltrated for 30 min in Ethanol/Technovit® 7200 (1:1) from HeraeusKulzer (Wehrheim,Germany) and in pure Technovit® 7200 at 4°C overnight. Afterwards the blood vessels were mounted with Technovit® 7230 in a histoform which was filled up with Technovit® 7200. Curing by light was done in an EXAKT 520 light chamber from EXAKT (Norderstedt, Germany) for 10 hours at white and 10 hours at blue light with.

For histochemistry, the polymerized blocks containing the blood vessels were adhered onto polycarbonate glass slides with Technovit® 7210 by curing under the precision adhesive press EXAKT 402 for 10 min. The curing process could be fastened by UV light.

Preparation for Immunohistochemistry and pre-labeled fluorescence

Technovit® 9100 was used for samples which were intended to be immunostained or already pre-labeled by fluorescence. The Technovit® 9100 kit contains different solutions which are described in this section. The preinfiltration solution consists of 1 g of hardener 1 and 200 ml of AL_2O_3 destabilized basic solution. The infiltration solution is a mixture of 1 g hardener 1, 20 g of PMMA-powder and 250 ml of destabilized basic solution. Stock solution A consists of 3 g hardener 1, 80 g PMMA-powder and 500 ml destabilized basic solution. Stock solution B consists of 4 ml hardener 2, 2 ml polymerization regulator and 50 ml destabilized basic solution.

After dehydration samples were incubated in Xylene (intermediate) from Merck (Darmstadt, Germany) for 45 min. Afterwards, samples were preinfiltrated in xylene/preinfiltration solution (1:1) for 45 min at RT, then for 45 min in absolute preinfiltration solution at RT and in addition for 45 min in preinfiltration solution at 4°C. Afterwards, the samples were incubated in infiltration solution overnight at 4°C. For polymerization 9 parts of stock solution A and 1 part of stock solution B were mixed. The sample was placed into an embedding form and the form was filled up with the polymerization solution. Air bubbles were removed by generating vacuum for a period of 10 min in the desiccator. The polymerization procedure was carried out at 4°C with -20°C cold thermal packs. For further preparation of the samples for immunohistochemistry, glass slides were coated with the adhesion agent ESPE™ Sil from 3M (St.Paul/Minnesota, USA). Afterwards, the polymerized samples were adhered on the slides in the same way as the samples for histochemistry (described above).

As for the histochemistry, Technovit® 7210 / EXAKT 402 was used to fasten curing of the adhesive with UV-light. The preparation of the samples for fluorescence was done by the instant adhesive for general purpose Loctite 401 from Henkel (Düsseldorf, Germany) to avoid bleaching of the GFP by exposing it to UV- light.

Sawing grinding and polishing

The diamond band saw EXAKT 300/310 was used to get sections of 100-400 µm. To further reduce the thickness, the samples were grinded and polished with the grinding system EXAKT 400CS. For determining the approximate thickness, four measurements had to be done with a digital micrometer.

Removal of Polymer prior to Immunohistochemistry

To enable antibody binding the polymerized plastic has to be removed on the surface of the sections. Therefore, the samples were incubated in Xylene two times for 20 min. Subsequently, samples were incubated in 2-methoxyethylacetate (2-MEA) for at least 24 h and finally in acetone for 5 s.

Histochemistry

For Masson Goldner staining, first the nuclei were stained with Weigert's Iron Hematoxylin (15 min) and washed under running tape water (5 min). Afterwards, they were stained in Masson Goldner Solution I (7 min) and washed by immersion into 0.2% acetic acid (5 times). After cleaning with distilled water, the sections were stained in Orange-G (5 min) and again immersed into 0.2% acetic acid (5 times). At least collagenous connective tissue was stained in light green, washed by immersion into 0.2% acetic acid and cleaned with distilled water.

For hematoxylin and eosin staining (H&E) sections were cleaned with aceton/ethanol (1:1) and the nuclei stained with Gill's Hematoxylin for 15 min. After bluing under running tap water (10 min), the tissue was placed into ethanol-dissolved eosin specialized for methacrylate embedding for 5 min and subsequently washed with distilled water.

Immunohistochemistry

After removing the polymerized plastics, the sections were demasked by autoclaving in 1 M citric buffer (pH 6) and washed with PBS. In the following steps the NOVA Detect DAB (3,3'-Diaminobenzidine) Quick strept AB-System from Dianova (Hamburg, Germany) was used. After each step, washing was done with PBS. Initially hydrogen peroxide solution was given on the tissue to block endogenous peroxidases (10 min). To prevent unspecific binding of the antibodies, samples were blocked by an Ultrablock (14 min) before incubating the sections with the primary polyclonal rabbit anti-human Von Willebrand factor antibody from Dako, 1:200 diluted in Dako antibody diluent (Hamburg, Germany). After 1.5 h the sections were washed and the secondary antibody (biotinylated goat anti-polyvalent plus) was pipetted onto the slide for 10 min. After washing, the tissue was incubated with streptavidin peroxidase for 10 min to bind to the biotin. At last, a mixture of DAB Plus Substrate (H_2O_2) and DAB Plus Chromogen (Diaminobenzidine) (50:1) was added. In presence of the streptavidin peroxidase a dark-brown DAB precipitate was developed.

Imaging by fluorescence intensity decay shape analysis microscopy (FIDSAM)

FIDSAM is a previously described method by Schleifenbaum et al. which is able to enhance the dynamic contrast of a fluorescence image of at least one order of magnitude. The technique is based on the analysis of the shape of the fluorescence intensity decay (fluorescence lifetime curve) and benefits from the fact that the decay patterns of typical fluorescence label dyes strongly differ from emission decay curves of auto-fluorescent sample areas. Therefore, FIDSAM enables the analysis of even low-concentrated fluorophores in tissue. Vessel sections were analyzed by FIDSAM for local GFP staining.

All images were recorded with a resolution of 50 nm per pixel and 5 mms integration time per pixel. However, for the application of FIDSAM, pixel-binning was used to collect enough photon events per pixel for robust statistics [14]. Here, we employed a binning of 5x5 pixels, which resulted in at least 3000 counts per pixel, up to 10000 counts in the brightest pixels. Furthermore, the chi^2-values of the fitting routine are normalized, so images with quite different intensities can be compared - provided the count statistics are sufficient (i.e >1000 counts per pixel). The resulting chi^2-map was then slightly blurred with a Gaussian algorithm (to smoothen out the rough edges of this more pixelated image) and used as a weighting filter for the original image. For a more detailed description of the method and the experimental setup see the original paper [14].

RESULTS

Sectioning and morphology

Sectioning proved to be laborious and technical challenging. Usually, only two or three sections could be produced per sample, because the sections are considerably thick. Specimen depletion was a concern. After grinding and polishing it was possible to reach a sample thickness smaller than 30 µm. But, the section thickness was found variable because of uneven section surfaces caused by a variable thickness of the adhesive. The stents struts consistently remained in situ and were never lost (Fig. 2-5). Sectioning artifacts such as scoring and folding were not found in the sections.

Histochemistry

The Masson Goldner staining consists of Weigert´s Hematoxylin, followed by Masson Goldner I solution, Orange-G and light green. After staining, collagenous connective tissue is typically green, the nuclei brown-black, the cytoplasm red, erythrocytes orange-red and muscular tissue pale red. In Figure 2 (Masson Goldner staining) the connective tissue of the stented arteries are stained in green, whereas the nuclei are barely apparent. The cytoplasm is not stained in red but in a color between yellow and orange, erythrocytes are more or less absent and the muscular tissue can be distinguished from the cytoplasm by a dark-orange color.

The H&E staining was carried out with Gill´s hematoxylin and eosin which stains the nuclei blue and the cytoplasm red. In Figure 3 staining of the stented arteries with H&E shows also blue colored nuclei and red cytoplasm.

Immunohistochemistry

The immunohistochemical detection of endothelial cells is limited to the von Willebrand factor because there are no other antibodies to porcine endothelial cells. The immunohistochemistry of Technovit 9100 embedded stented arteries against the endothelial specific Von Willebrand factor is shown in Figure 4. Herein the brown chromogen indicating the bound antibody can be found at the first cell layer at the site of the lumen and additionally in the area of connective tissue. The Von Willebrand factor is released by endothelial cells in the presence of physical stress and is able to bind to collagenous tissue which might explain the brown chromogen in the area of connective tissue[15].

Imaging by fluorescence intensity decay shape analysis microscopy (FIDSAM)

To analyze the emission of the fluorescent label fluorescence and auto-fluorescence we implanted GFP reporter gene-coated stents ex vivo into porcine arteries for 24 hours. The analysis of the stented arterial tissue with normal fluorescence microscopy and same settings (exposure, acquisition time) shows no difference in fluorescence intensity between coated or non-coated stents (Fig. 5, at the top). Using FIDSAM, the image is now analyzed pixel by pixel, fitting a mono-exponential decay function to the measured fluorescent emission curves. Low chi^2 error values correspond to highly matching fits, i.e. the fluorescent label, and are denoted black or gray. Areas with a high contribution of auto-fluorescence (typically exhibiting multi-exponential decay behavior) deviate from the mono-exponential reference function, therefore show high chi^2 error values and are indicated in white (Fig. 5, middle).

To further visualize these results, the pixels of the original image and the chi^2 values are combined to a FIDSAM-image, in such a manner that areas with high auto-fluorescence are suppressed while areas with mainly fluorescent label remain unaffected (Fig. 5, at the bottom).

While the fluorescence intensity images of coated and non-coated stents are barely different (Fig. 5, top), the FIDSAM analysis reveals that the strong fluorescence of the non-coated stents can be assigned to auto-fluorescence, showing high chi^2 values, which therefore vanishes in the FIDSAM image (Fig. 5, middle and bottom). In contrast, the arteries of GFP reporter gene-coated stents show specific fluorescence, derived from GFP-expression in the tissue (Fig. 5, middle, bottom).

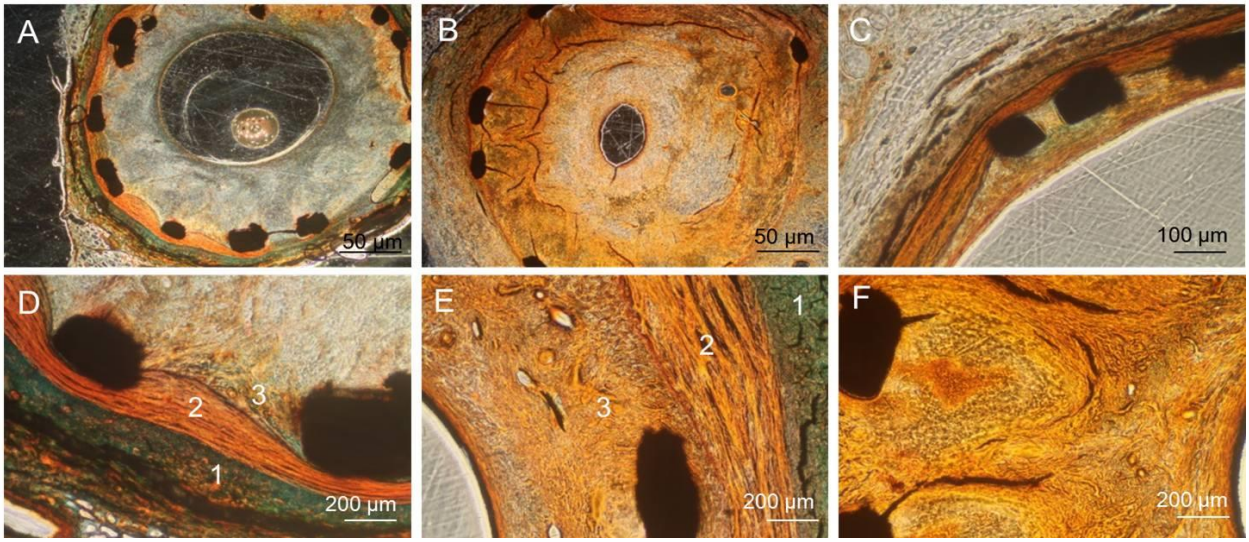

Fig. 2 A-F. Histochemical staining with Masson Goldner: In D and E, connective tiussue (green) is marked with 1, muscular tissue (dark-orange) with 2 and the cytoplasm (between yellow and orange) with 3 (A and B; 1:60 magnification, C; 1:100 magnification, D, E and F; 1:200 magnification).

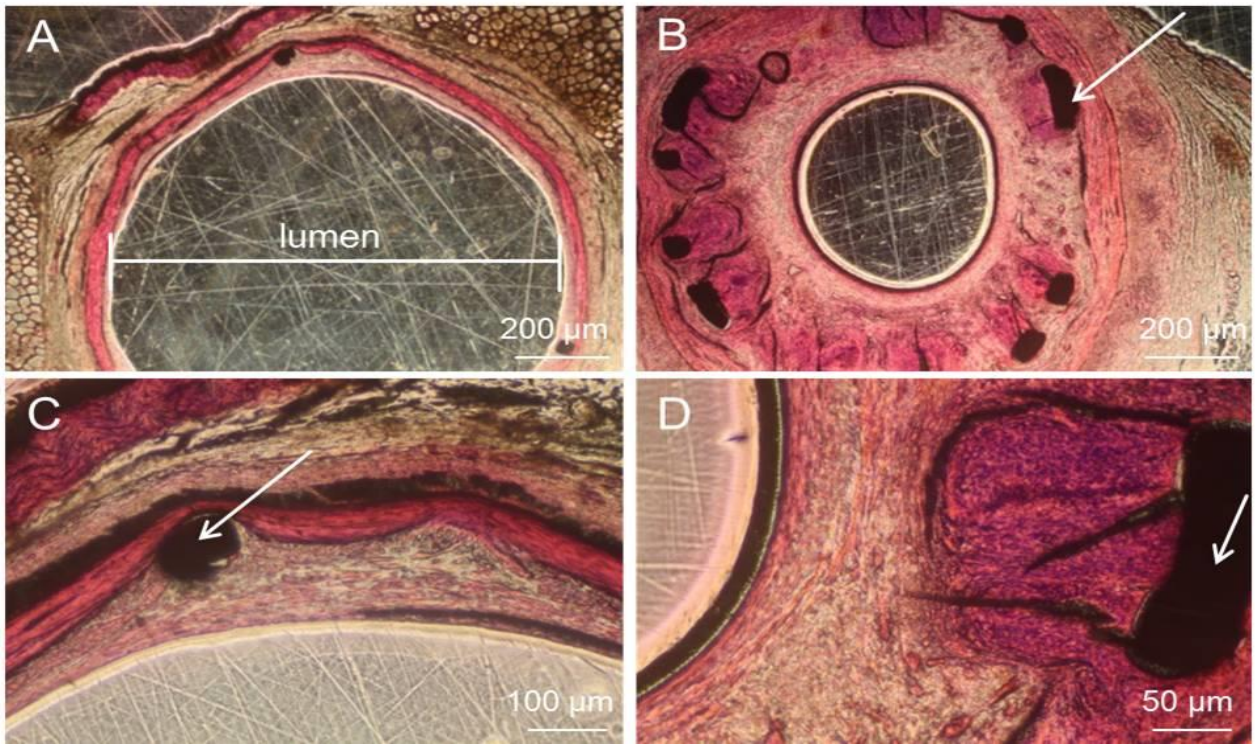

Fig. 3 A-D. Histological staining with Gill´s hematoxylin and eosin: The lumen is indicated with the white bar in A, Stent strut is indicated with a white asterisk (A and B; 1:60 magnification, C; 1:100 magnification and D; 1:200 magnification).

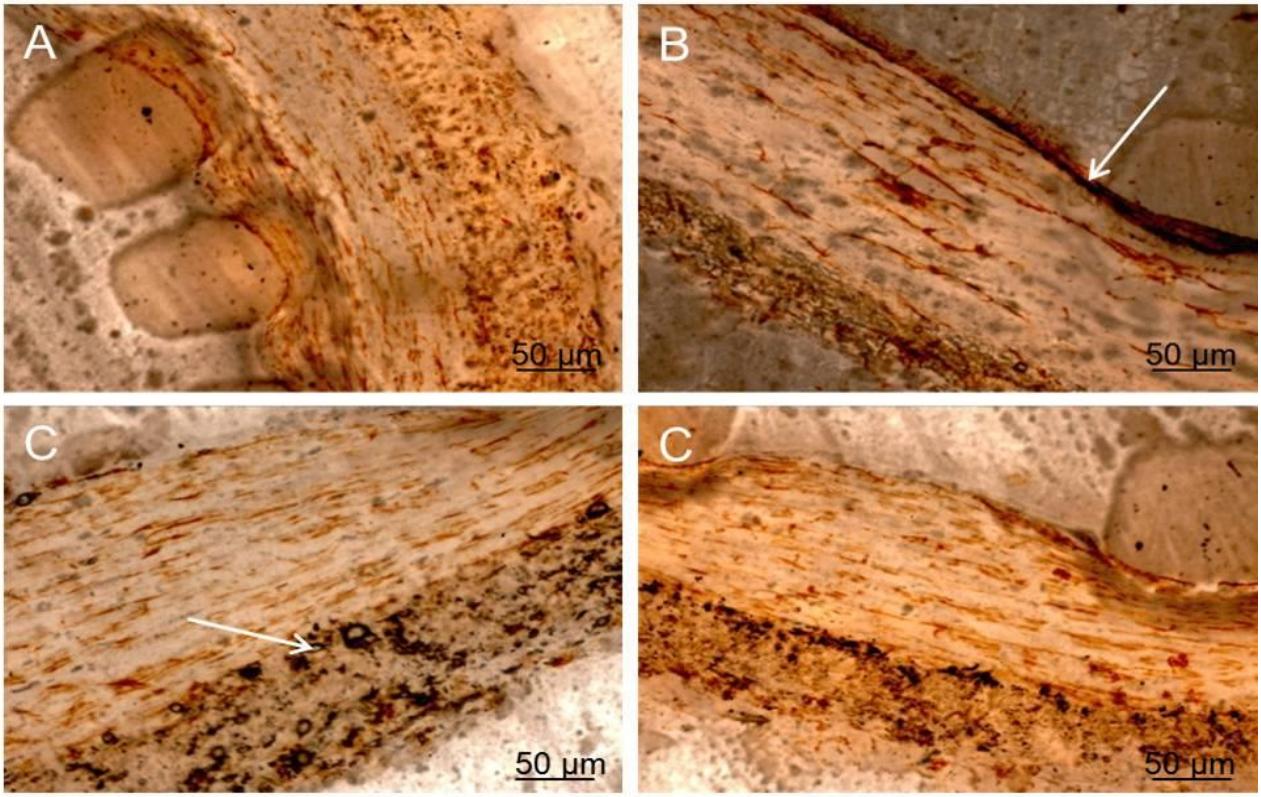

Fig. 4 A-D. Immunohistochemical labelling with the von Willebrand factor; Brown chromogen indicating the bound antibody can be found at the first cell layer at the site of the lumen and additionally in the area of connective tissue, marked by asterisk (A, B, C and D; 1:200 magnification).

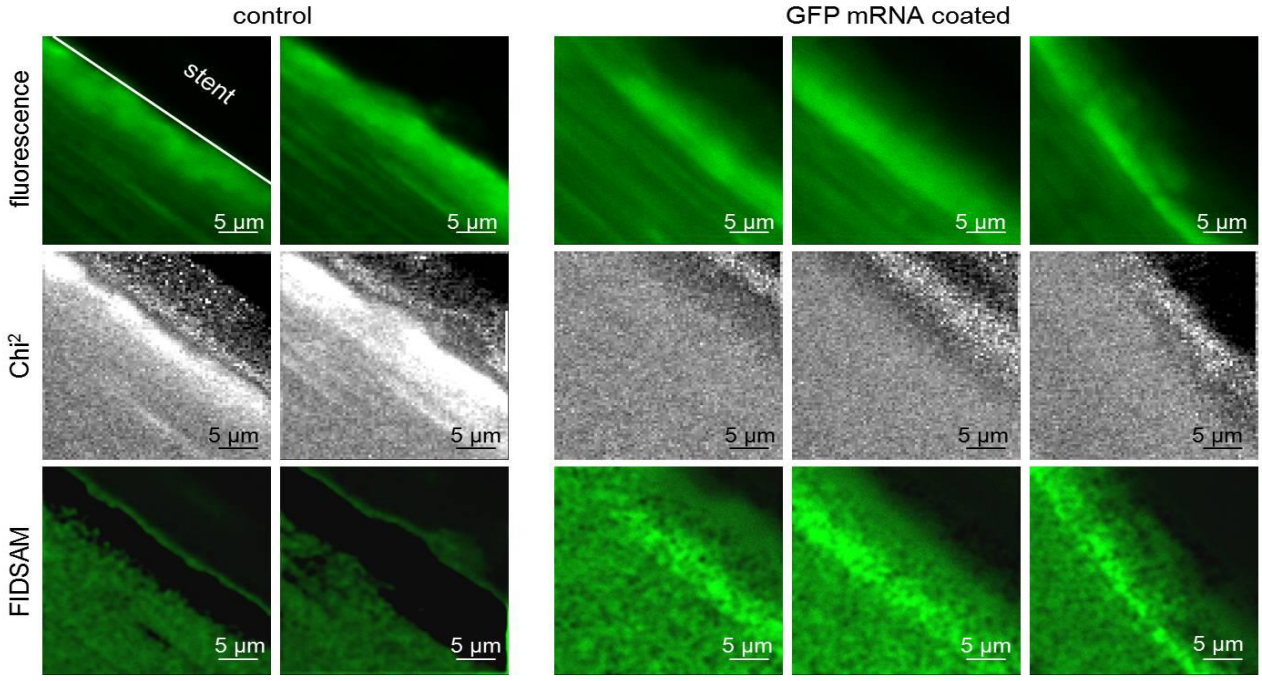

Fig. 5. Fluorescence intensity decay shape analysis (FIDSAM) of arterial cross sections with GFP reporter gene coated stents and non-coated stents; (left) control with non-coated stents implanted in coronary artery, (right) GFP reporter gene coated stents implanted in coronary artery. The right upper corner in each image indicates the stent strut (marked with a white line). The bar indicates a length of 5 µm. The images of coated and non-coated stents were always taken under the same conditions and instrument settings

DISCUSSION

During recent decades considerable advances were achieved in intravascular interventions for the treatment of arterial disease. In particular, drug eluting stents (DES) – although not as perfect as primarily expected - partly replaced BMS [16]. The efficacy of BMS was severely hampered by proliferating VSMCs and the resultant neointimal hyperplasia [17]. The advent of DES in 2002 have since then revolutionized interventional cardiology. As valid for every new technology also DES have some restraints for example the delayed endothelialization. Meanwhile, several new stent coatings, like the CD34 antibody coated endothelial progenitor cell (EPC) capture stent, are under verification in patient studies [18]. Results of several clinical studies indicate that the ideal stent is not yet available, and therefore industry as well as the scientific community keep on working hard on the development of more sophisticated solutions for the post-DES era [19]. To better understand the vascular response to stent implantation and to identify the possible targets for enhanced clinical outcomes, the histologic preparation of stented arteries has to be improved substantially.

In this paper we want to summarize the methodologies and results of different embedding methods, each used for different examination techniques. With the use of different methacrylates we were able to reach a sample thickness of smaller than 30 µm without artifacts such as scoring or folding. Furthermore, no stent strut was lost or out of place. The production of multiple sections proved to be laborious and technical challenging. Additional grinding and polishing often lead to sample loss. Lately, we were able to produce also sections with a thickness of around 5 µm (data not shown). Good results concerning the thickness of the sections and a decreased sample loss need a lot of practical experience.

Rippstein et al. also described the need of practical experience [10]. They compared tungsten carbide blade and saw and grinding sectioning methods. In their view the use of tungsten carbide blade gave more consistent results. They reported that the saw and grinding method reveals thicker sections compared to the tungsten carbide blade. They also observed sectioning artifacts such as scoring and folding with tungsten carbide blade and not with saw and grinding [10].

Our sections were suitable for both immunohistochemical and histochemical staining. Thereby, immunohistochemical staining could only be performed when the sections were deplasticized. The embedding of fluorescently labeled tissue revealed that it is important to use methacrylates that polymerize without light, to circumvent possible bleaching of the

fluorophores. We could show that our embedding methods did not damage the fluorophores.

The evaluation of arterial cross sections with FIDSAM technology gave new, very important insights into the interpretation of fluorescently labeled tissue. The observation of the arterial cross sections by conventional fluorescence microscopy displayed the same fluorescence intensity, whether the stents were coated with GFP reporter gene or not. Due to the analysis of the cross sections by FIDSAM, we were able to show that GFP fluorescence was only detectable when stents were coated with GFP reporter gene and only auto-fluorescence was observed when stents were not coated. Our findings indicated that the auto-fluorescence in arterial tissue is very high. Further, the auto-fluorescence is very high in general and increases at positions, where the stent strut squeezes the tissue. To analyze the release kinetics or diffusion of fluorescent labeled drugs coated on a stent is therefore hindered. One has to be aware that the analysis of the drug distribution with fluorescently labeled drugs is only possible with technologies like FIDSAM.

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