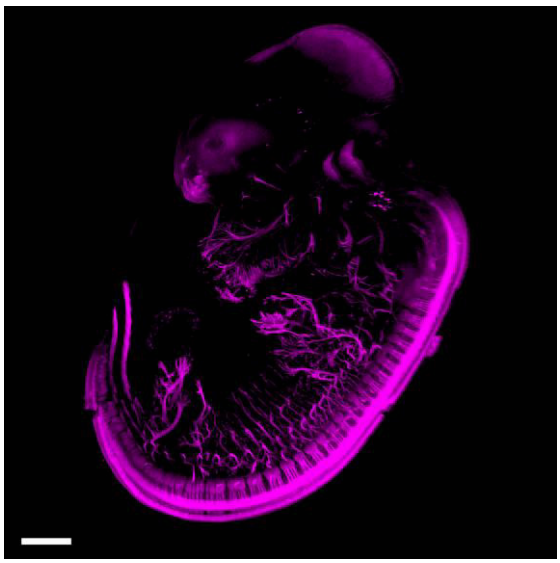
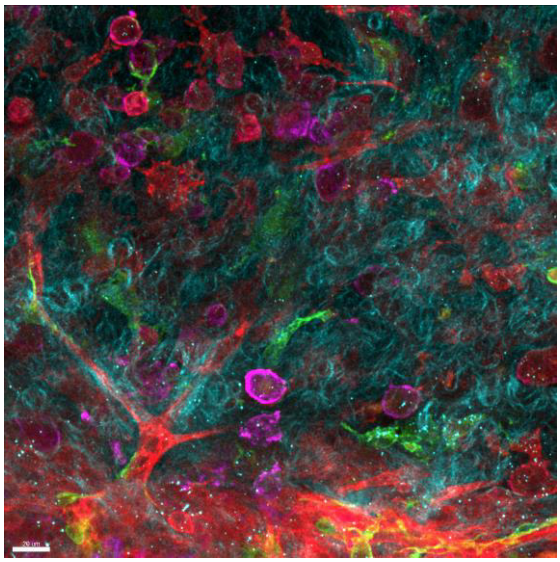
Imaging fibrotic responses to injury – New methods from development to pathology





Simon Christ

Doctoral Thesis

Aus dem Comprehensive Pneumology Center (CPC) / Institute of Lung Biology and Disease (ILBD), Helmholtz Zentrum München
Vorstand: Dr. Antje Brand

Imaging fibrotic responses to injury – New methods from development to pathology

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Affidavit

Simon Christ

Hereby I declare,
that the submitted thesis entitled *Imaging fibrotic responses to injury – New methods from development to pathology* is my own work. I have only used the sources indicated and have not made unauthorised use of services of a third party. Where the work of others has been quoted or reproduced, the source is always given.

I further declare that the submitted thesis or parts thereof have not been presented as part of an examination degree to any other university.

Munich, 30.10.2020 — Simon Christ

Place, date — Signature of doctoral candidate

Abbreviations

3DISCO	3D imaging of solvent cleared organs
DT	diphtheria toxin
DTR	diphtheria toxin receptor
ECM	extracellular matrix
En1	engrailed 1
ENF	En1-lineage-naive fibroblast
EPF	En1-lineage-past fibroblast
GFP	green fluorescent protein
HIF1-α	hypoxia-inducible factor 1 alpha
IVM	intravital microscopy
MIP	maximum intensity projection
MSLN	mesothelin
MSOT	multispectral optoacoustic tomography
PC	panniculus carnosus
PDPN	podoplanin
RFP	red fluorescent protein
SHG	second harmonic generation
α-SMA	alpha-smooth muscle actin

List of publications

Publication I:

Jiang, D.*, Correa-Gallegos, D.*, **Christ, S.***, Stefanska, A., Liu, J., Ramesh, P., Rajendran, V., De Santis, M.M., Wagner, D.E. & Rinkevich, Y. (2018). Two succeeding fibroblastic lineages drive dermal development and the transition from regeneration to scarring. *Nature cell biology*, *20*(4), 422. * contributed equally

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Publication III:

Correa-Gallegos, D., Jiang, D., **Christ, S.**, Ramesh, P., Ye, H., Wannemacher, J., Kalgudde Gopal, S., Yu, Q., Aichler, M., Walch, A., Mirastschijski, U., Volz, T. & Rinkevich, Y. (2019). Patch repair of deep wounds by mobilized fascia. *Nature*, 576(7786), 287-292.

1. Introduction

New modalities in fluorescence imaging can drive and reveal fundamental aspects in biomedical research. With the advent of new technologies, machinery and protocols, researchers nowadays have a powerful toolbox to study biology on a microscopic scale with astonishing resolution at both spatial and temporal levels. Microscopy is one of the few analytical methods that produces accurate spatial information on the molecule or structure of interest. Increasing speed, resolution and performance come with more complex sample preparation, more sophisticated technical setup and more complicated image analysis. Consequently, a gap has emerged between cutting-edge technology and biologists routinely using microscopic techniques for their research (Power & Huisken, 2019). Scientists heaving knowledge on both, the technical as well as on the biological aspects can bridge this gap and bring the latest advancements to the bench.

1.1 Aims

The aim of this dissertation was to implement new imaging techniques for studying biological processes during development and in context of organ disease. More specifically, we were interested in general fibrotic responses following injury, in both skin and internal organs. Scarring is thought of as a general outcome of quick but defective and excessive tissue repair (Greenhalgh, 2007). The failed repair mechanism results in impaired functionality, restrictions in motion and contractility, discomfort, pain and even death (Zeisberg & Kalluri, 2012). Regenerative research aims to preserve or re-activate mechanisms that initially can form a fully functional organism during development. The regenerative capacity is constantly reduced during adult life in most vertebrates and a scarring phenotype is characteristic of tissue/organ injury in postnatal life. Trying to reverse this process, from scarring-to-regeneration, presupposes a thorough picture of the developmental stage, the steady- and diseased state. The proverbial picture of a biological state is the main discipline of microcopy as such and imaging in a broader sense. Microscopy offers two different ways of usage in a scientific context. Firstly, it is used to observe biology at a level usually hidden for the human eye. Upon observation of an interesting feature, researchers usually compare different stages, states or conditions and consequently develop and formulate biological questions. The saying “seeing is believing” means that the visual perception is extremely well evolved in humans and our brain is highly confident on visual input. Secondly, it is used to document the presence or absence, the amount, the behavior or dynamics of cells, molecules and

structures of interest. It therefore enables one to prove or disprove previously formulated hypotheses.

1.2 Advances in fluorescent microscopy

A whole plethora of different microscopy techniques have been developed over the last fifty years based on those two basic operational modes. Fluorescence microscopy has proven to be the most sensitive and versatile method. Most of the popular advances have been using this physical principle of the absorption of light and subsequent emission of light that has lower energy than the incident light. Be it multi-photon microscopy (Strickler, et al., 1990), super-resolution microscopy (Hell & Wichmann, 1994) or light-sheet microscopy (Huisken, et al., 2004) and all their derivatives. They all share the common feature to allow for imaging in all three spacial dimensions. As we live in a three-dimensional world, biology should be looked at accordingly. A sample that was physically sectioned in order to make it accessible for light microscopy offers only a fraction of the original informative content. Everything above or below a thin and finite volume is ignored. Especially supracellular structures and networks suffer from this preparation. Not surprisingly, neuroscience was always in the frontline of developing new optical methods to detect far spanning neuronal connections (Dodt et al., 2007; Ertürk et al., 2011). True 3D imaging is achieved via optical sectioning and successive 3D reconstruction of the single planes, leaving the original sample intact. A general limitation is the restricted penetration depth of light in un-dissected biological tissue. Absorption and scattering of incident and emitted light make it very difficult to look deep inside the sample.

One strategy to overcome this limitation is the use of long wavelength light in the far-red or infrared region. The low scattering and absorption in this spectral range (700 – 1300 nm) is utilized in multi-photon microscopy. Two photons of roughly half the energy (or three photons of one third the energy in case of three-photon excitation) that is normally needed are used to excite a certain fluorophore. High peak power, pulsed lasers guarantee sufficient probability for the simultaneous absorption to occur. Less energetic light can penetrate deeper into tissue and it also reduces toxic effects in live preparations.

The second strategy to increase penetration depth is to render the sample transparent. The idea is to clear for scattering and absorbing molecules and remove transitions in refractive indices within the sample (Susaki & Ueda, 2016). A variety of tissue clearing methods for different tissues and sample sizes are described (Richardson & Lichtman, 2015).

1.3 Sample preparation

In the publications presented in this dissertation we used both, multi-photon microscopy and light-sheet microscopy of optically cleared tissues to image a broad range of samples in high resolution. Due to the incompatibility of the multi-photon objective with organic clearing solvents used in our tissue clearing protocol, we could not use both methods in combination. Their application however demands appropriate sample preparation. We therefore developed a strategy to conjoin both preparation protocols, simultaneously. In this way both methods can be applied on the same sample if needed. The first part involves immunolabeling of the protein of interest. Antibodies and fluorescent probes are allowed to diffuse into the fixed tissue using an adopted protocol from Belle et al. (Belle et al., 2014). Depending on the size of the sample this process is completed within 3-14 days. After immunostaining the samples can be subjected to whole mount 3D multi-photon microscopy or directly be treated for tissue clearing. The clearing protocol is based on the 3DISCO procedure (Ertürk et al., 2012). In the last step of the protocol the sample is immersed in an organic solvent, which is also the imaging environment. These samples are typically imaged using a light-sheet or confocal microscope.

1.4 Imaging of live tissue

Imaging of living tissues adds another dimension to microscopic imaging. The temporal dimension can reveal dynamics and behavioral aspects of cells or proteins of interest in live samples that are otherwise lost or distorted during subsequent histochemical protocols. It can be utilized to visualize, for example, how cells react to a stimulus or treatment, how they behave in different conditions or at different developmental stages, how cells move with respect to thier location and how their morphology changes over time. This additional information comes with the price of additional measures that need to be considered. The main aspect is viability of the sample. Physiologic conditions can be ensured by the use of an environmental chamber. Using far-red excitation and reasonable laser power can minimize toxic effects (Foyer et al., 1994; Rieder & Khodjakov, 2003). Live cell imaging mostly makes use of cells growing in a dish under controlable conditions and in a format that is compatible to microscops (Stevens & Allan, 2003; Ettinger & Wittman, 2014). But since cells change morphology, behavior and activity on plastic (Vlodavsky et al., 1980), they are best imaged in their native habitat as a whole *ex vivo* or even *in vivo*. Only then exogenous stimuli and their influence on the cells behavior can be prevented. Consequently, intravital microscopy (IVM) models for diverse organs have been developed and are used to study immunology (Sumen et al., 2004), oncology (Condeelis & Segall, 2003), renal and hepatic disease (Molitoris & Sandoval, 2005), and regeneration (Pineda et al., 2015). IVM is the closest observation scenario of biology as it occurs in real life. But it is also the most challenging one. There are multiple factors, such as anesthesia, vital parameters, stabilization against movement, accessibility, that need to be taken in to account in order to obtain useful data.

In the publications presented in the dissertation and in a submitted manuscript (Jiang, Christ, Correa-Gallegos, Ramesh et al., *Cell Stem Cell*, in review, not included) we developed a setup for multi-photon live imaging of whole organs *ex-vivo* and an intravital microscopy model of wound healing.

2. Two succeeding fibroblastic lineages drive dermal development and the transition from regeneration to scarring

2.1 Introduction

Mammalian back skin reacts differently to injuries in fetal life compared to postnatal stages. Strikingly, during early embryogenesis mammals can heal skin with no obvious scars, whereas later dermal injuries are patched with a dense collagen plug, a scar (Mast et al., 1992). This scarless to scarring transition occurs beginning of the last trimester of embryonic development. It was shown before that early expression of the transcription factor engrailed 1 (En1) defines a fibroblastic lineage with cell intrinsic fibrogenic potential (Rinkevich et al., 2015). The central aim of this study was to link the emergence of scars with the expansion of the scar prone En1-lineage-past fibroblast (EPF) lineage in the murine dorsal back skin. Elucidating the transition of scarless regeneration to skin scarring holds clinically relevant potential in the prevention of scars and their treatment.

There was the need for an imaging technique, which faithfully recapitulates the spatial and temporal location of a fibroblastic subpopulation in a developing embryo on a cellular level. Heaving a complex distribution of the cells of interest both on a compartment and tissue level, we decided to use an optical clearing method combined with immunolabeling of a transgenic reporter system. Hence, we used the double-fluorescent reporter mouse line Rosa26$^{mT/mG}$ (B6.129(Cg)-*Gt(ROSA)26Sor*$^{tm4(ACTB-tdTomato,-EGFP)Luo}$) and crossed it to an En1 cre-driver line (*En1*$^{tm2(cre)Wrst}$;En1^{Cre}). Double transgenic offspring universally express membranous red fluorescent protein (RFP) and upon cre dependent recombination switches to permanent membrane bound GFP expression. Cre^{+} embryos were collected at different time points and subjected to whole mount immunolabeling followed by a solvent based optical clearing method. Immunolabeling of fluorescent proteins became necessary because the native fluorescence of fluorescent proteins is heavily quenched by dehydration and is not stable in organic solvents (Belle et al., 2014). It furthermore gave us the opportunity to use far-red fluorophores with higher penetration depth and lower scattering compared to conventional fluorophores. Due to the limited working distance of the objectives in our confocal microscopy setup, only half of the embryo was scanned. We also tested light-sheet microscopy of the sample, which in principle suits the optically cleared sample type better, but it was almost impossible to mount the fragile embryos to the holder and if successful, cellular resolution could not be achieved.

The second major contribution to this project was the live imaging setup. From whole embryo 3D imaging we knew, that EPFs populate the dorsal dermis in an arcing fashion from anterior to posterior and spreading from the dermomyotome ventrally. We were interested in the migration characteristics of EPFs during the ENF-to-EPF replacement. Therefore, we went on to establish a confocal live-imaging setup. Since the cells of interest are located directly underneath the developing epidermis, which is very thin at this developmental stage, confocal microscopy with a penetration depth of a few tens of micrometers was sufficient and multi-photon microscopy was not needed. In order to stably fix the sample during image acquisition, we embedded the embryo in a collagenous 3D matrix. Physiological conditions were ensured with the use of an environmental chamber heated to 37°C and 5% CO_2 supplemented air. In order to track single cells, we used the less phototoxic and far-red excitable live stain SiR-DNA (Spirochrome AG, Switzerland). As cell density increases drastically during the ENF-to-EPF replacement, the membrane bound GFP signal was not sufficient to identify cell boundaries and the additional nuclear stain became necessary. Furthermore, cell detection algorithms work significantly better on nuclear signal compared to a membrane signal. By combining these approaches, we were able to obtain good GFP/RFP signal for identification of fibroblast lineage and generated good quality tracks from the nuclear signal for cell tracking. We were able to maintain healthy cells over a period of 24 hours. The setup enabled us to show that EPFs migrate into spaces freed by ENFs. The live data also showed the population of the dermis by EPFs in a dorsally and laterally directed migration trajectory.

Combined, the whole mount 3D imaging of optically cleared embryos and confocal live-imaging of developing embryos allowed us to study the scarless regeneration to scar formation process in great detail with state of the art spatial and temporal resolution. The appealing image of the optically cleared embryo was further selected as cover image for the Nature Cell Biology journal issue.

2.2 Contribution

- Sample preparation, 3D immunolabeling and clearing of E12.5 embryo (Fig. 2f).
- Confocal live imaging $En1^{Cre};R26^{mT/mG}$ embryo (Fig. 2j; Video 2).
- 3D representations form live imaging (24h) of anterior region in $En1^{Cre};R26^{mT/mG}$ embryo and maximum intensity projection (MIP) of whole embryo (Suppl Fig 4).
- Support in planning and analysis of experiments. Helped in the preparation of figures and writing of the respective sections.

2.3 Publication I

Jiang, D.*, Correa-Gallegos, D.*, Christ, S.*, Stefanska, A., Liu, J., Ramesh, P., Rajendran, V., De Santis, M.M., Wagner, D.E. & Rinkevich, Y. (2018). Two succeeding fibroblastic lineages drive dermal development and the transition from regeneration to scarring. *Nature cell biology*, *20*(4), 422. * contributed equally

https://doi.org/10.1038/s41556-018-0073-8

3. Surgical adhesions in mice are derived from mesothelial cells and can be targeted by antibodies against mesothelial markers

3.1 Introduction

Postsurgical adhesions are fibrotic bridges that form between abdominal organs or internal organs and the peritoneal wall. They are an outcome of a trauma to the serosa lining induced by abdominal surgery or peritoneal dialysis and bare great clinical implications and a burden on healthcare systems. The molecular mechanism and cellular origin underlying postsurgical adhesions has been vague. In Tsai et al. we identified podoplanin (PDPN) and mesothelin (MSLN) expressing mesothelial cells as the main drivers of adhesion formation in mice trough hypoxia-inducible factor 1 alpha (HIF1-α) dependent pathways.

First, we used whole mount multi-photon imaging combined with 3D immunolabeling to show surface marker expression in mesothelial cells and their contribution to abdominal adhesions. Since the mesothelium is a monocellular layer covering several body cavities and all internal organs, it is quite difficult to fully visualize its morphological aspects. Once the sample is sectioned for histology, the two-dimensional layer is reduced to one-dimensional line, which makes it difficult to examine its distribution and coverage on a surface. We therefore decided to image the samples (healthy peritoneum and adhesion) as a whole head-on with a volumetric multi-photon scan. We immunostained the sample prior to imaging to visualize the expression of the surface markers PDPN, MSLN and a marker for myofibroblasts alpha-smooth muscle actin (α-SMA). Using a multi-photon microscopic approach, we were able to image the whole surface with a penetration depth of a few hundred micrometers deep into the tissue.

Abdominal adhesions were induced using a combination of ischemic buttoning and abrasion of opposing surfaces. With this model we were able to reproducibly induce adhesions between the peritoneal wall and internal organs. The membranous expression of PDPN revealed cobblestone morphology of healthy mesothelium on the peritoneal wall. In contrast, MSLN expression was low in healthy mesothelium and absent within the tissue. The expression of α-SMA under steady-state conditions was restricted to vascular smooth muscle cells. Following injury, $PDPN^{+}$ mesothelial cells lose their cell-cell contacts and adopt an activated morphology. They stay the predominant cell population within the injured surface and expand to a multilayered structure. In case of an injury the mesothelial surface marker MSLN gets locally upregulated and overlaps with PDPN expression. Therefore, we

identified PDNP+ MSLN+ mesothelial cells as the main drivers of adhesion formation. Notably, the expression of fibroblast-associated α-SMA was upregulated following injury.

In order to study the accessibility of the target cells we switched to a different method. We established an in-vivo immunolabeling protocol using fluorescently labeled antibodies. This method allowed the visualization of protein expression throughout the entire injury site and local protein expression at the time if injury. The idea was to only label the early response of MSLN expression on the injured peritoneal surface up to 30 minutes after injury. We labeled an anti-MSLN antibody with the fluorescent tag IRDye 800CW (LI-COR Biotechnology, Lincoln, NE, USA) and injected the conjugate after surgery into the peritoneal cavity. After 30 minutes the mouse was sacrificed and organs were collected. After the samples were rendered transparent with an organic solvent-based clearing method, they were subjected to light sheet microscopy. Using this technique, we found local upregulation of MSLN in injured mesothelial cells. The expression of MSLN was localized to the site of injury and already apparent 30 minutes after induction.

With this experimental setup, we intended to use multispectral optoacoustic tomography (MSOT), a noninvasive intravital-imaging approach, to image early MSLN expression in injured mesothelium in live mice. This could be a valuable diagnostic detection tool of a clinically relevant biomarker for postsurgical adhesions. Unfortunately, the available resolution of the method was not sufficient at the time.

Taken together, we showed that local upregulation of MSLN in mesothelial cells upon injury is the main driver of adhesion formation in the abdominal cavity.

3.2 Contribution

- Animal model of post-surgical adhesions and administration of labeled antibody. 3D light sheet imaging of optically cleared sample and 3D rendering of data (Fig. 3a,b; Fig. S17; Movie S1,S2).
- Whole mount immunostaining (PDPN, MSLN, α-SMA) of healthy and injured peritoneum 24h post surgery and 3D multi-photon imaging (Fig. S3).
- Helped designing the in-vivo immunolabeling model
- Helped preparing the figures and wrote respective material and methods section.

3.3 Publication II

Tsai, J. M., Sinha, R., Seita, J., Fernhoff, N., Christ, S., Koopmans, T., Krampitz, G.W., McKenna, K.M., Xing, L., Sandholzer, M., Sales, J.H., Shoham, M., McCracken, M., Joubert, L-M., Gordon, S.R., Poux, N., Wernig, G., Norton, J.A., Weissman, I.L. & Rinkevich, Y. (2018). Surgical adhesions in mice are derived from mesothelial cells and can be targeted by antibodies against mesothelial markers. *Science translational medicine*, *10*(469), eaan6735.

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4. Patch repair of deep wounds by mobilized fascia

4.1 Introduction

Mammalian wounds are quickly patched with dense collagen plugs by invading fibroblasts. But the origin of fibroblasts has been elusive. The current dogma of immigrating dermal fibroblasts and their subsequent *de novo* synthesis of ECM is challenged by our findings on a previously undescribed mobile fascia.

In this project, microscopy played a pivotal role in both, identifying and studying the dynamic mobility of fascia during wound healing. First we found that the connective tissue layer, located underneath the panniculus carnosus (PC), is highly enriched in fibroblasts by using a high resolution multi-photon scan of the dorsal skin in a neonatal double transgenic mouse ($En1^{Cre}$;$R26^{mT/mG}$). Scar-prone engrailed1-past fibroblasts (EPF) were arranged in continuous monolayers ranging from interscapular fat patch to the hip region. Strikingly, we found open passages to the dermis in parts where nerve fibers and blood vessels transit to the dermis. Even though there is a direct connection from fascia to dermis their respective cellular compositions are quite different. We found higher number of fibroblastic, endothelial and lymphatic cells in the fascia and a similar count for immune and nerve cells. The challenge was to find a way to image the 1,5 cm whole dermis (epidermis, dermis, panniculus carnosus, fascia) in a conceiving way with high resolution that would allow morphological identification of single cells. We successfully did so by carefully excising the dorsal back skin, separating it from underlying skeletal muscle and embedding it into a layer of agarose upside down. Here it was important to use enough agarose to stably fixate the sample without covering it in order not to interfere with the optical light path. The high resolution of the multi-photon scan allowed us to zoom in to any region across the sample and identifying localized cellular composition, morphologies and large-scale organization. Furthermore, we established a superficial wounding model, in which we removed only epidermis and dermis, leaving the PC and the underlying superficial fascia intact. Using this model, we could show that EPFs can traverse the PC upwards into the dermis in response to injury.

With the use of multi-photon microscopy, we were also able to obtain second harmonic generation (SHG) signal from fibrillar collagens and therefore visualize the ECM architecture in a label-free manner. SHG signal in fascia showed a coiled structure indicative for relaxed fibers. Comparing the structure of immature fascia matrix to corrugated and tensed dermal collagen network showed significant differences in ECM architecture. We also used SHG signal to track fascia in a wound healing scenario. Here, we developed an incubation

chamber to allow live imaging of fascia biopsies for an extended period of time. This custom-made chamber permitted the water-dipping objective of an upright microscope to be fully immersed in the media, while still sealing the chamber against loss of sterility, humidity, CO_2 atmosphere and heat. At the same time, it needed to provide degrees of freedom for normal operation of the stage and focus. Using this newly developed setup, we were able to visualize fascia movements based on SHG signal up to 30 hours and showed locomotion with a rate of 11.4 µm/h. This corresponds to about 2 mm in 7 days of a normal wound healing process. We went on to investigate role of EPFs and proliferation in the process of fascia movement. Using the same experimental setup, we imaged fascia biopsies with depleted EPFs. This was achieved using cre sensitive diphtheria toxin receptor (DTR) expression and exposure to diphtheria toxin (DT). Fascia depleted of EPFs showed no movement. In a second experiment we supplemented the media with proliferation inhibitor Etoposide and saw no significant differences in migration behavior.
In conclusion, we showed fascia architecture and cellular composition in neonatal back skin using a large volume multi-photon scan. Furthermore, we visualized EPF driven fascia movements using a custom-made incubation chamber.

4.2 Contribution

- 3D live imaging (30h) and quantification of fascia movement in full thickness skin biopsy (Fig. 3d,e).
- Volumetric multi-photon image of superficial fascia in $En1^{Cre};R26^{mT/mG}$ mice (Extended Data Fig. 2f) and large-area 3D tile-scan and high magnification zoom-in images of whole neonatal skin (Extended Data Fig. 2g,h,i).
- 3D imaging of fascia and panniculus carnosus in $En1^{Cre};R26^{Rainbow}$ mice (Extended Data Fig. 2j).
- Superficial wounding in back skin of $En1^{Cre};R26^{mT/mG}$ mice and 3D tile-scan 3 dpw (Extended Data Fig. 2k).
- 25 h live imaging and quantification of contraction of DT treated skin biopsy from $En1^{Cre};R26^{iDTR}$ mice and Etoposide treated biopsy (Extended Data Fig. 9d,e,I,h).
- Design and implementation of used incubation chamber
- Support in planning and analysis of experiments. Helped in writing of the respective sections.

4.3 Publication III

Correa-Gallegos, D., Jiang, D., Christ, S., Ramesh, P., Ye, H., Wannemacher, J., Kalgudde Gopal, S., Yu, Q., Aichler, M., Walch, A., Mirastschijski, U., Volz, T. & Rinkevich, Y. (2019). Patch repair of deep wounds by mobilized fascia. *Nature*, 576(7786), 287-292.

https://doi.org/10.1038/s41586-019-1794-y

5. Summary

In the publications included in this dissertation we successfully implemented a broad range of cutting-edge microscopy techniques into basic science. We demonstrated how improvements in fluorescence microscopy can accelerate biomedical research by providing high resolution imaging of biological processes in space and time. The obtained multi-dimensional data allowed us to study fibrotic responses from development to adulthood and from homeostasis to pathology. The observations made with these techniques had a great impact on finding the right research questions and answering them with convincing visual data.

In the first publication we used whole mount 3D-imaing of optically cleared embryos to show the emergence of scar prone fibroblasts during development. Furthermore, we could unravel their migration behavior by confocal live imaging. Taken together we linked the scarless-to-scarring transition in developing mammals with the given priority for this fibroblastic subpopulation.

In the second publication we elucidated the mechanism and cellular origin of abdominal adhesions, a postsurgical fibrotic pathology. We utilized whole mount 3D-imaging of optically cleared samples in combination with an in-vivo immunolabeling method to show the local upregulation of a targetable surface marker in injured mesothelium. Furthermore, we identified the cellular origin and initiation process with the use of volumetric multi-photon microscopy.

In the third publication presented in this dissertation, we used large-area 3D multi-photon scanning to show that superficial fascia in murine back skin is enriched in scar prone fibroblasts. We used the same method to further characterize the cellular composition and ECM structure and found significant differences compared to dermis. We could also show that superficial fascia is the main source for wound fibroblasts and ECM in dermal wound repair. We developed a new custom-made imaging chamber for multi-photon live imaging to show the dynamics of mobile fascia. Our finding will challenge the current dogma of invading fibroblasts and *de novo* synthesis of ECM.

The custom made chamber and a novel intravital multi-photon microscopy model for cutaneous wound healing was extensively used to show dynamics of cell migration during wound healing in a submitted manuscript that is currently under revision and therefore not included in this dissertation.

6. Zusammenfassung

In den Publikationen, die in dieser Dissertation enthalten sind, zeigen wir erfolgreich die Umsetzung modernster Mikroskopietechniken in die Grundlagenforschung. Wir zeigen wie neue Entwicklungen in der Fluoreszenzmikroskopie die biomedizinische Forschung durch eine hohe Auflösung in Raum und Zeit beschleunigen und erleichtern können. Die gewonnenen multi-dimensionalen Daten erlaubten es uns, fibrotische Prozesse von der Entwicklung bis zum Erwachsenenalter und von der Homöestase bis zur Pathologie zu untersuchen. Die mit diesen Techniken gewonnenen Erkenntnisse hatten einen großen Einfluss darauf, die richtigen Forschungsfragen zu finden und diese mit überzeugenden visuellen Daten zu beantworten.

In der ersten Veröffentlichung haben wir ein 3D-Bildgebungsverfahren von optisch geklärten Embryonen verwendet, um das Aufkommen von narbenbildenden Fibroblasten während der Entwicklung zu zeigen. Darüber hinaus konnten wir ihr Migrationsverhalten durch konfokale Mikroskopie von vitalen Proben abbilden. Wir haben damit den Übergang von der narbenlosen zu narbenreichen Wundheilung in der späten Entwicklungsphase von Säugetieren mit der erhöhten Präsenz dieser fibroblastischen Unterpopulation verknüpft.

In der zweiten Publikation haben wir den Mechanismus und die zelluläre Herkunft von Bauchverwachsungen, einer häufigen fibrotischen Pathlogie nach Bauchhöhlenoperationen, aufgeklärt. Dafür haben wir ein 3D-Bildgebungsverfahren von optisch transparenten Proben in Kombination mit einer in-vivo Immunmarkierungsmethode verwendet. Wir konnten damit die lokale Hochregulation eines Oberflächenmarkers im verletzten Mesothel zeigen, der mittels Immuntherapie beeinflussbar ist. Darüber hinaus haben wir den zellulären Ursprung und Initiationsprozess mit Hilfe der volumetrischen Mehrphotonen-Mikroskopie identifiziert.

In der dritten Publikation, die in dieser Dissertation vorgestellt wurde, haben wir mittels einer großflächigen 3D-Aufnaheme gezeigt, dass die oberflächliche Faszie in der murinen Rückenhaut mit narbenbildenden Fibroblasten angereichert ist. Wir verwendeten die gleiche Methode, um die zelluläre Zusammensetzung und die ECM-Struktur weiter zu charakterisieren und fanden signifikante Unterschiede im Vergleich zu der darüberliegenden Haut. Des Weiteren konnten wir zeigen, dass dieses Bindegewebe die Hauptquelle für verschiedene Zelltypen und ECM bei der dermalen Wundheilung ist. Wir haben eine neue, maßgeschneiderte Inkubationskammer für die Mehrphotonen-Mikroskopie entwickelt, um die Mobilität der Faszie zu zeigen. Die gängige Meinung, dass Fibroblasten nach einer Verwundung in das verletzte Gewebe einwandern und dort neue extrazelluläre Matrix synthetisieren, wird durch unsere Ergebnisse in Frage gestellt.

Die oben erwähnte Inkubationskammer und ein neuartiges intravitales Wundheilungmodell für die Mehrphotonen-Mikroskopie wurden in einem weiteren Manuskript umfassend behandelt, das sich derzeit in review befindet und daher nicht Bestandteil dieser Arbeit ist. Diese beiden innovativen Methoden wurden entwickelt, um die Dynamik der Zellmigration während der Wundheilung zu zeigen.

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