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Visualization and manipulation of repair and regeneration in biological systems using light

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“By the means of telescopes, there is nothing so far distant but may be represented to our view; and by the help of microscopes, there is nothing so small as to escape our inquiry, hence there is a new visible world discovered to the understanding. ”

— Robert Hooke, 1665 —

Declaration

I, Stefan Michael Klaus Kalies, born on 09/16/1987, herewith declare that for the writing of this habilitation thesis "Visualization and manipulation of repair and regeneration in biological systems using light" the following statements are true:

1. This thesis has been composed entirely by myself.

2. I have not used any sources and aids other than those indicated by me.

3. My contribution to each included publication is explicitly indicated in the publication list. I confirm that appropriate credit has been given within this thesis where reference has been made to the work of others.

I am aware of and understand the university's policy on plagiarism and I certify that this thesis is my own work and has not been submitted in support of another degree or qualification from this or any other university.

Hannover, May 2022

Stefan Michael Klaus Kalies

Colophon
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Abstract

Tissue repair after an injury is a fundamental process in biomedicine. It can involve regeneration, which uses new growth to restore tissue function. The interest in repair and regeneration is motivated by the desire to treat injuries and diseases and has attracted researchers for centuries. In the last decades, it evolved in the field of regenerative medicine, which has the ultimate goal of providing strategies for regenerating human cells, tissues, or even organs, for instance, via engineering principles.

Already since the first experiments on regeneration by Abraham Trembley, novel findings in biomedicine, repair, and regeneration have been enabled or accompanied by research in optics, for example, on the development of novel microscopy techniques. Nowadays, novel optical techniques are advancing, which allow to understand the role of single cells in tissue repair processes. Moreover, repair processes within cells can be visualized and manipulated. Ultimately, optics can provide enabling techniques for regenerative therapies. This habilitation thesis aims to present several of these advances.

On a single cell level, femtosecond laser nanosurgery was used to target specific intracellular structures during concurrent imaging *in vitro*. The relation of femtosecond laser nanosurgery to the cell state and cellular staining was investigated. Manipulation of single Z-discs in cardiomyocytes using a femtosecond oscillator laser system was accomplished, which allows to better elucidate the role of a single Z-disc in cardiomyocyte function. In particular, measurements on cell survival, (calcium-) homeostasis, and morphology yielded only minor deviations from control cells after single Z-disc ablation. A reduction in force generation was elucidated via traction force microscopy and gene expression level changes, for instance, an upregulation of α-actinin were examined. Additionally, light-based systems to influence single cells in their alignment or to trigger single cells, for example, to activate other cells via optogenetics were applied.

On the tissue scale, imaging via confocal microscopy or multiphoton microscopy has been applied for various contexts of regenerative approaches. Furthermore, a fiber-based imaging approach, which could later be used for longitudinal imaging *in vivo* and builds upon a fluorescence microscope system and an imaging fiber bundle in combination with reconstruction via a neural network, was developed. As another imaging strategy, an abdominal imaging window served to image the mouse liver *in vivo* via multiphoton microscopy in successive imaging sessions. Manipulation in tissue was applied in colonoids, which resemble the structure of the colon on an *in vitro* scale, and revealed different cell dynamics dependent on the location of the damage. In particular, activation of the Wnt signaling pathway after crypt damage was observed. Cell ablation via a femtosecond laser amplifier system during concurrent two-photon microscopy was also established during *in vivo* liver imaging to study micro-regenerative processes.

Furthermore, laser-based delivery processes with novel materials or in the context of genome editing using CRISPR/Cas9 technology were investigated as enabling technologies for regenerative medicine.

In conclusion, this thesis addresses the question of how optics can help to illuminate future directions in research on tissue repair and regeneration, as well as, regenerative therapies by addressing (longitudinal) imaging in a complex environment, sophisticated cell-manipulation strategies, and the application of novel materials for laser-based delivery.

Zusammenfassung

Die Reparatur von Gewebe nach einer Verletzung ist ein grundlegender Prozess in der Biomedizin. Sie kann einen Regenerationsprozess beinhalten, bei dem neues Gewebewachstum zur Wiederherstellung der Gewebefunktionen verwendet wird. Forschung an diesen Prozessen ist durch den Wunsch motiviert, Verletzungen und Krankheiten behandeln zu können. Daraus ist das Feld der regenerativen Medizin entstanden, das Strategien zur Regeneration von Zellen, Gewebe oder Organen entwickelt.

Bereits seit den ersten Regenerationsversuchen von Abraham Trembley wurden neue Erkenntnisse zur Reparatur und Regeneration durch Fortschritte in der Optik ermöglicht, wie die Entwicklung neuer Mikroskopietechniken. Heutzutage werden optische Techniken entwickelt, die die Rolle einzelner Zellen bei Gewebereparaturprozessen aufdecken können. Außerdem können Reparaturprozesse innerhalb von Zellen visualisiert und manipuliert werden. Letztendlich kann Optik auch Techniken für regenerative Therapien bereitstellen. In dieser Habilitationsschrift werden diese Fortschritte vorgestellt.

In einzelnen Zellen wurde die Femtosekundenlaser-Nanochirurgie eingesetzt, um spezifische intrazelluläre Strukturen *in vitro* zu manipulieren. Dabei wurde der Zusammenhang zwischen dem Manipulationsprozess, dem Zellzustand und der zellulären Färbung untersucht. Die Manipulation einzelner Z-Scheiben in Herzmuskelzellen mit einem Femtosekunden-Oszillator-Lasersystem ermöglichte es, die Rolle einer einzelnen Z-Scheibe in der Funktion der Herzmuskelzelle besser zu verstehen. Messungen der Vitalität, der (Calzium-) Homöostase und der Morphologie ergaben nur geringe Abweichungen von Kontrollzellen nach Ablation einer einzelnen Z-Scheibe. Mit Hilfe der Traction-Force-Mikroskopie wurde eine Verringerung der Krafterzeugung festgestellt. Eine veränderte Genexpression, zum Beispiel eine Hochregulation von α-Actinin wurde beobachtet. Darüber hinaus wurden lichtbasierte Systeme eingesetzt, um Zellen einerseits in ihrer Ausrichtung zu beeinflussen oder andererseits über Optogenetik zur Aktivierung anderer Zellen zu bringen.

Auf der Gewebeskala wurde die Bildgebung mittels konfokaler Mikroskopie oder Multiphotonenmikroskopie in verschiedenen regenerativen Ansätzen eingesetzt. Es wurde ein faserbasierter Bildgebungsansatz entwickelt, der später für die longitudinale Bildgebung *in vivo* verwendet werden kann. Dieser basiert auf einem Fluoreszenzmikroskopsystem und einem bildgebenden Faserbündel in Kombination mit der Rekonstruktion über ein neuronales Netzwerk. Als weitere Bildgebungsstrategie diente ein abdominales Bildgebungsfenster für Multiphotonenmikroskopie der Mausleber *in vivo* in aufeinanderfolgenden Bildgebungssitzungen. Die Manipulation von Gewebe wurde auch in Kolonoiden angewandt, welche die Struktur des Dickdarms auf einer *in vitro*-Skala nachbilden und zeigte in Abhängigkeit der Lokalisation der Schädigung unterschiedliche Einflüsse. Es wurde eine Aktivierung des Wnt-Signalwegs nach Schädigung der Krypte beobachtet. Die Zellablation mittels eines Femtosekundenlaser-Verstärkersystems bei gleichzeitiger *in vivo* Multiphotonenmikroskopie der Leber wurde etabliert, um mikro-regenerative Prozesse zu untersuchen.

Weiterhin wurden laserbasierte Zellmanipulationsprozesse für die Zelltransfektion im Zusammenhang mit der Genom-Editierung mittels CRISPR/Cas9-Technologie untersucht. Ebenfalls wurden dabei neue Materialien für diesen Prozess ausgetestet.

Zusammenfassend geht es in dieser Arbeit um die Frage, wie Optik Forschung zur Gewebereparatur und -regeneration sowie zu regenerativen Therapien in der Zukunft unterstützen kann. Dabei wurde die Bildgebung in komplexen Umgebungen, hochentwickelte Zellmanipulationsstrategien und die Anwendung neuartiger Materialien für die laserbasierte Zellmanipulation evaluiert.

Contents

Publication list 1

Publications are listed in reverse chronological order. Co-authors are indicated by an asterisk.

1.1 Peer-reviewed publications, which are part of this habilitation

Key publications of this thesis

1. S. Donath, L. Angerstein, L. Gentemann, D. Müller, A. E. Seidler, C. Jesinghaus, A. Bleich, A. Heisterkamp, M. Büttner, and **S. Kalies**. 'Investigation of Colonic Regeneration via Precise Damage Application Using Femtosecond Laser-Based Nanosurgery'. *Cells* **11** (7) (2022)

   Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

2. D. Müller, S. Donath, E. G. Brückner, S. Biswanath Devadas, F. Daniel, L. Gentemann, R. Zweigerdt, A. Heisterkamp, and **S. Kalies**. 'How Localized Z-Disc Damage Affects Force Generation and Gene Expression in Cardiomyocytes'. *Bioengineering* **8** (12) (2021)

   Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

3. P. Heeger, J. Harre, A. Warnecke, D. Mueller, **S. Kalies***, and A. Heisterkamp*. 'Probing interneuronal cell communication via optogenetic stimulation'. *Translational Biophotonics*, e202100002 (2021)

   Contribution: partial study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

4. D. Müller, T. Klamt, L. Gentemann, A. Heisterkamp, and **S. Kalies**. 'Evaluation of laser induced sarcomere micro-damage: Role of damage extent and location in cardiomyocytes'. *PloS one* **16** (6), e0252346 (2021)

   Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

5. J. Bahlmann, N. Madrahimov, F. Daniel, D. Theidel, D. E. DeTemple, M. Buettner, A. Bleich, A. Haverich, A. Heisterkamp*, and **S. Kalies***. 'Establishment of a guided, in vivo, multi-channel, abdominal, tissue imaging approach'. *Scientific Reports* **10** (1), 1–9 (2020)

   Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

6. D. E. DeTemple, S. Cammann, J. Bahlmann, M. Buettner, A. Heisterkamp, F. Vondran*, and **S. Kalies***. 'Longitudinal imaging and femtosecond laser manipulation of the liver: How to generate and trace single-cell-resolved micro-damage in vivo.' *PloS one* **15**, e0240405 (2020)

   Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

7. D. Müller, D. Hagenah, S. Biswanath, M. Coffee, A. Kampmann, R. Zweigerdt, A. Heisterkamp, and **S. Kalies**. 'Femtosecond laser-based nanosurgery reveals the endogenous regeneration of single Z-discs including physiological consequences for cardiomyocytes'. *Scientific Reports* **9** (1), 1–10 (2019)

Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

8. D. Hagenah, A. Heisterkamp, and **S. Kalies**. 'Effects of cell state and staining on femtosecond laser nanosurgery'. *Journal of Biophotonics* **11** (7), e201700344 (2018)

Contribution: study design, coordination and supervision, experiments, data analysis, drafting and writing of the manuscript

9. B. Bošnjak, M. Permanyer, M. K. Sethi, M. Galla, T. Maetzig, D. Heinemann, S. Willenzon, R. Förster, A. Heisterkamp, and **S. Kalies**. 'CRISPR/Cas9 Genome Editing Using Gold-Nanoparticle-Mediated Laserporation'. *Advanced Biosystems* **2** (11), 1700184 (2018)

Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

10. N. Saklayen*, **S. Kalies***, M. Madrid, V. Nuzzo, M. Huber, W. Shen, J. Sinanan-Singh, D. Heinemann, A. Heisterkamp, and E. Mazur. 'Analysis of poration-induced changes in cells from laser-activated plasmonic substrates'. *Biomedical Optics Express* **8** (10), 4756–4771 (2017)

Contribution: study design, coordination and supervision, experiments, data analysis, management of the cooperation, drafting and writing of the manuscript

Additional publications of this thesis

11. W. Shen, **S. Kalies**, M. Madrid, A. Heisterkamp, and E. Mazur. 'Intracellular Cargo Delivery Induced by Irradiating Polymer Substrates with Nanosecond-Pulsed Lasers'. *ACS Biomaterials Science and Engineering* (2021)

Contribution: partly study design, data analysis, management of the cooperation, writing of the manuscript

12. F. Manstein, K. Ullmann, C. Kropp, C. Halloin, W. Triebert, A. Franke, C.-M. Farr, A. Sahabian, A. Haase, Y. Breitkreuz, M. Peitz, O. Brüstle, **S. Kalies**, U. Martin, R. Olmer, and R. Zweigerdt. 'High density bioprocessing of human pluripotent stem cells by metabolic control and in silico modeling.' *Stem Cells Translational Medicine* **10**, 1063–1080 (2021)

Contribution: multiphoton imaging

13. S. Heene, S. Thoms, **S. Kalies**, N. Wegner, P. Peppermüller, N. Born, F. Walther, T. Scheper, and C. A. Blume. 'Vascular network formation on macroporous polydioxanone scaffolds'. *Tissue Engineering Part A* (2021)

Contribution: help with confocal imaging

14. H. Ichanti, S. Sladic, **S. Kalies**, A. Haverich, B. Andrée, and A. Hilfiker. 'Characterization of Tissue Engineered Endothelial Cell Networks in Composite Collagen-Agarose Hydrogels'. *Gels* **6** (3), 27 (2020)

Contribution: multiphoton imaging

15. I. Takayama, N. Kondo, **S. Kalies**, A. Heisterkamp, and M. Terakawa. 'Myoblast adhesion and proliferation on biodegradable polymer films with femtosecond laser-fabricated micro through-holes'. *Journal of Biophotonics* **13** (7), e202000037 (2020)

Contribution: initial establishment of the in vitro experiments

16. P. Brooks, T. zur Bruegge, E. C. Boyle, **S. Kalies**, S. N. Villarreal, A. Liese, A. Bleich, and M. Buettner. 'CD14 and ALPK1 Affect Expression of Tight Junction Components and Proinflammatory Mediators upon Bacterial Stimulation in a Colonic 3D Organoid Model'. *Stem Cells International* **2020** (2020)

Contribution: help with confocal imaging

17. H. C. Janßen, N. Angrisani, **S. Kalies**, F. Hansmann, M. Kietzmann, D. P. Warwas, P. Behrens, and J. Reifenrath. 'Biodistribution, biocompatibility and targeted accumulation of magnetic nanoporous silica nanoparticles as drug carrier in orthopedics'. *Journal of Nanobiotechnology* **18** (1), 1–18 (2020)

Contribution: multiphoton imaging

18. A. Becker, T. Lehrich, **S. Kalies**, A. Heisterkamp, and A. Ngezahayo. 'Parameters for Optoperforation-Induced Killing of Cancer Cells Using Gold Nanoparticles Functionalized With the C-terminal Fragment of Clostridium Perfringens Enterotoxin'. *International Journal of Molecular Sciences* **20** (17), 4248 (2019)

Contribution: help with initial study design and derivation of the methodology

19. B. Andrée, H. Ichanti, **S. Kalies**, A. Heisterkamp, S. Strauß, P.-M. Vogt, A. Haverich, and A. Hilfiker. 'Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels'. *Scientific Reports* **9** (1), 1–11 (2019)

Contribution: multiphoton imaging

20. S. Johannsmeier, P. Heeger, M. Terakawa, **S. Kalies**, A. Heisterkamp, T. Ripken, and D. Heinemann. 'Gold nanoparticle-mediated laser stimulation induces a complex stress response in neuronal cells'. *Scientific Reports* **8** (1), 1–12 (2018)

Contribution: help with initial study design and derivation of the methodology

1.2 Peer-reviewed publications, which are not part of this habilitation

These publications have been published during the doctoral thesis or before, or do not fit the scope of this habilitation.

1. M. Schünemann, K. Sperlich, K. Barnscheidt, S. Schöpa, J. Wenzel, **S. Kalies**, A. Heisterkamp, H. Stolz, O. Stachs, and B. Hage. 'Balanced Heterodyne Brillouin Spectroscopy Towards Tissue Characterization'. *IEEE Access* **10**, 24340–24348 (2022)

2. T. F. Zur Bruegge, A. Liese, S. Donath, **S. Kalies**, M. Kosanke, O. Dittrich-Breiholz, S. Czech, V. N. Bauer, A. Bleich, and M. Buettner. 'Intestinal Organoids in Colitis Research: Focusing on Variability and Cryopreservation.' *Stem Cells International* **2021**, 9041423 (2021)

3. L. Gentemann*, **S. Kalies***, M. Coffee, H. Meyer, T. Ripken, A. Heisterkamp, R. Zweigerdt, and D. Heinemann. 'Modulation of cardiomyocyte activity using pulsed laser irradiated gold nanoparticles'. *Biomedical Optics Express* **8** (1), 177–192 (2017)

4. **S. Kalies***, G. C. Antonopoulos*, M. S. Rakoski, D. Heinemann, M. Schomaker, T. Ripken, and H. Meyer. 'Investigation of biophysical mechanisms in gold nanoparticle mediated laser manipulation of cells using a multimodal holographic and fluorescence imaging setup'. *PLoS One* **10** (4), e0124052 (2015)

5. **S. Kalies**, S. Keil, S. Sender, S. C. Hammer, G. C. Antonopoulos, M. Schomaker, T. Ripken, H. M. Escobar, H. Meyer, and D. Heinemann. 'Characterization of the cellular response triggered by gold nanoparticle–mediated laser manipulation'. *Journal of Biomedical Optics* **20** (11), 115005 (2015)

6. M. Schomaker, D. Killian, S. Willenbrock, D. Heinemann, **S. Kalies**, A. Ngezahayo, I. Nolte, T. Ripken, C. Junghanß, H. Meyer, et al. 'Biophysical effects in off-resonant gold nanoparticle mediated (GNOME) laser transfection of cell lines, primary-and stem cells using fs laser pulses'. *Journal of Biophotonics* **8** (8), 646–658 (2015)

7. M. Schomaker, D. Heinemann, **S. Kalies**, S. Willenbrock, S. Wagner, I. Nolte, T. Ripken, H. M. Escobar, H. Meyer, and A. Heisterkamp. 'Characterization of nanoparticle mediated laser transfection by femtosecond laser pulses for applications in molecular medicine'. *Journal of Nanobiotechnology* **13** (1), 1–15 (2015)

8. D. Begandt, A. Bader, G. C. Antonopoulos, M. Schomaker, **S. Kalies**, H. Meyer, T. Ripken, and A. Ngezahayo. 'Gold nanoparticle-mediated (GNOME) laser perforation: a new method for a high-throughput analysis of gap junction intercellular coupling'. *Journal of Bioenergetics and Biomembranes* **47** (5), 441–449 (2015)

9. **S. Kalies**, D. Heinemann, M. Schomaker, L. Gentemann, H. Meyer, and T. Ripken. 'Immobilization of gold nanoparticles on cell culture surfaces for safe and enhanced gold nanoparticle-mediated laser transfection'. *Journal of Biomedical Optics* **19** (7), 070505 (2014)

10. **S. Kalies***, D. Heinemann*, M. Schomaker, H. M. Escobar, A. Heisterkamp, T. Ripken, and H. Meyer. 'Plasmonic laser treatment for Morpholino oligomer delivery in antisense applications'. *Journal of Biophotonics* **7** (10), 825–833 (2014)

11. **S. Kalies**, L. Gentemann, M. Schomaker, D. Heinemann, T. Ripken, and H. Meyer. 'Surface modification of silica particles with gold nanoparticles as an augmentation of gold nanoparticle mediated laser perforation'. *Biomedical Optics Express* **5** (8), 2686–2696 (2014)

12. **S. Kalies***, T. Birr*, D. Heinemann, M. Schomaker, T. Ripken, A. Heisterkamp, and H. Meyer. 'Enhancement of extracellular molecule uptake in plasmonic laser perforation'. *Journal of Biophotonics* **7** (7), 474–482 (2014)

13. D. Heinemann, M. Schomaker, **S. Kalies**, T. Ripken, and H. Meyer. 'Cell manipulation by gold nanoparticle mediated laser transfection'. *Biomedical Engineering-Biomedizinische Technik* **59** (2014)

14. D. Heinemann*, **S. Kalies***, M. Schomaker, W. Ertmer, H. M. Escobar, H. Meyer, and T. Ripken. 'Delivery of proteins to mammalian cells via gold nanoparticle mediated laser transfection'. *Nanotechnology* **25** (24), 245101 (2014)

15. D. Heinemann, M. Schomaker, **S. Kalies**, M. Schieck, R. Carlson, H. M. Escobar, T. Ripken, H. Meyer, and A. Heisterkamp. 'Gold nanoparticle mediated laser transfection for efficient siRNA mediated gene knock down'. *PloS one* **8** (3), e58604 (2013)

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17. **S. Kalies**, K. Kuetemeyer, and A. Heisterkamp. 'Mechanisms of high-order photobleaching and its relationship to intracellular ablation'. *Biomedical Optics Express* **2** (4), 805–816 (2011)

1.3 Patent applications

1. A. Heisterkamp, **S. Kalies**, D. Müller. "Analysis method for m-RNA of individually light-irradiated cells" - WO2020069918A1

Optics to shed light on damage, repair, and regeneration

2

In 1665, Robert Hooke was the first to describe a microorganism, the microfungus *Mucor*, in his book *Micrographia* [1, 2]. *Micrographia* illustrated about 60 microscopic observations and also was the first book with microscopy drawings. Only about eleven years later, in 1676, Antoni van Leeuwenhoek discovered bacteria in week-old "pepper water" [2, 3]. Hooke and Leeuwenhoek were driven by the development of their own microscopes, for instance, via smaller optics. Further observations were submitted by Leeuwenhoek in his letters to the Royal Society. Among these was the description of the freshwater polyp Hydra in 1704, which paved the way for the first observations of regeneration by Abraham Trembley [4–7]. Trembley worked with a microscope made by John Cuff. He dissected Hydra into two parts and discovered a remarkable regenerative capacity: Two new *Hydras* developed, linking regeneration to generation [7].

Optics allowed the first observation of regeneration by Abraham Trembley using a microscope made by John Cuff.

Since this time, the investigation of repair and regenerative processes and the use of optics to understand these have intensified. In the early nineteenth century, it was again the microscope, which helped to develop the cell theory by Matthias Schleiden for plants and Theodor Schwann for animal tissue [8]. At the same time, Ernst Abbe developed important knowledge about microscopy [9, 10] that today serves as the basis for all modern microscopes. This, together with the invention of fluorescent dyes by Adolf von Bayer and the first dye-based cellular fluorescence staining by Stanislaus von Prowazek enabled seminal findings in biomedicine and regeneration.

In the last fifty years, the application of optics has powered novel findings in biology. The discovery and use of green fluorescent protein [11, 12], the pioneering of flow cytometry [13], or the development of pulsed lasers, which can be used for multiphoton microscopy [14] are only some milestones from this era. Further, optics have also found their way into medical applications. In addition to basic imaging modalities such as microscopy or endoscopy, newer techniques such as optical coherence tomography have evolved [15]. Additionally, optical methods such as photothermal therapy have been applied as therapeutic strategies, for example, in cancer [16–18]. Nowadays, physicists still aim to improve the visualization of biological processes. The invention of super-resolution microscopy techniques has enabled visualizations at the subcellular level that go beyond the diffraction limit derived by Ernst Abbe [19]. Additionally, non-visualization applications of optics have evolved on a cellular level: The optical stimulation of cells through optogenetics is one example [20]. Moreover, the use of optics in biomedicine is influenced by novel computational resources such as neural networks, which can help to enhance and interpret data [21].

Modern optics finds widespread use in biomedicine, involving techniques such as flow cytometry, high-resolution and high-penetration depth microscopy, endoscopy, photodynamic or photothermal applications, or optogenetics.

Modern optics and regeneration

Can modern optics still shed more light on damage, repair, and regeneration? The ultimate aim, to understand repair and regeneration, is to investigate damage on a single-cell level, to analyze cells in their complex environment, to link phenotype to genotype, to investigate their transcriptome and proteome, and to learn about intercellular interactions. Optics can, as seen from history, help to visualize these factors and processes. But it can also serve to manipulate them on a cellular level to gain a better understanding. This habilitation thesis will address both of these ways to better understand cellular repair and regeneration.

This habilitation addresses modern optics to better understand tissue and cell repair and regeneration.

Before this chapter will lay the ground and discuss some important aspects of repair and regeneration, with a special emphasis on their optical analysis, it requires to define these terms. The term regeneration is today used in a variety of contexts. For instance, in public, the terms "ecosystem regeneration" or "regenerate earth" became popular. The ideas of regeneration are nowadays also linked to sustainability, defining "regenerative sustainability" as a holistic worldview that addresses the whole system and involves "co-creation" [22]. This depiction is already inspired by biology and shows important aspects, which need to be considered describing regeneration: It happens on different scales, the whole system has to be considered, and it involves "creation" [23].

A biomedical definition, which is used within this habilitation, can be derived based on Krafts et al. [24]: After tissue damage, repair is the restoration and reconstruction of its architecture and function. It involves regeneration, which uses new growth (generation) to restore the tissue to its original state or replacement (such as scarring) if regeneration is not possible. Often both, regeneration and replacement, occur during repair. To nevertheless take into account the different scales of the whole system, the term repair needs to be extended to include damage to single cells. If single cells reach their original state after damage, for example, membrane damage, this habilitation will use the terms repair and cellular remodeling.

Repair and **regeneration** address the whole biological system and happen on different scales. **Repair** is the general **restoration of architecture and function**. In tissue, **regeneration involves new growth** (generation) of cells. In single cells, repair and remodeling are used as terms to describe the healing of single-cell damage.

2.1 What determines regenerative capacity?

The ability to restore and reconstruct damaged tissue is widespread among animals. However, the capacity for restoration and reconstruction varies significantly. Hydra, as mentioned before, can regenerate from the smallest pieces. In contrast, salamanders exhibit limb, tail, eye, jaw, and heart regeneration [25], whereas mammals usually cannot regenerate appendage tissue. However, there are some exceptions. For instance, Bedelbaeva et al. demonstrated that mice lacking expression of the cyclin dependent kinase inhibitor protein p21 protein that is involved in cell cycle regulation through the cyclin kinase pathway showed an ear hole closure phenotype associated with a strong regenerative response [26]. In humans, the most popular

regenerating organ is the liver. These examples give rise to the question: What determines regenerative capacity - and how can we investigate it? This formulation is accompanied by more specific questions, as we are also interested in the origin of regenerating cells, their specification, and the process of regeneration initiation [25].

The cellular origin is highly dependent on the regenerating tissue (see Figure 1). In the first case of regeneration, differentiated cells produce more cells through proliferation. In the mammalian liver, for example, the response to injury involves the proliferation of hepatocytes. In the second case, stem cells are involved in the regenerating tissue. These are defined by their ability to self-renew and the capacity to produce differentiated cell types [25, 27, 28]. A popular example of such tissue is the mammalian intestine. Stem cells reside in the intestinal crypts and self-renew the intestine every four to five days [29]. Additionally, dedifferentiation to a progenitor cell type or transdifferentiation into another cell type can be involved in the regenerative response [25, 30]. Dedifferentiation is a process that takes place in nerve regeneration: Schwann cells dedifferentiate, proliferate and remyelinate the regenerated axons [31]. Transdifferentiation naturally is a stepwise process from a differentiated cell, which undergoes first dedifferentiation and second differentiation, into the new lineage [30]. As an example, the pigment epithelial cells of the newt lens undergo transdifferentiation during regeneration [32]. Often, in particular in the case of limb, tail, or digit regeneration, the cell mass, which orchestrates the regenerative response is called blastema and is mainly composed of undifferentiated cells [33].

Often, regeneration is linked to stem cells. These are defined by their ability to self-renew and the capacity to produce differentiated cell types.

For fate specification of the cells during regeneration, their surrounding environment needs to be taken into account. This includes, for example, the existence of protein gradients or gene expression patterns. Renewing tissue is often marked by different levels of Wnt expression. Wnt is a growth factor that is highly conserved in evolution and mainly conveys proliferation signals between cells. In the mammalian intestine, for example, Wnt expression decreases from crypt bottom to top [35]. Several studies have identified Wnt signaling to be of crucial importance in fate specification during regeneration across species [36–40]. This is not surprising, as Wnt also has a broad role in embryonic development [40, 41]. Next to Wnt, further growth factors or tissue-specific genes can play a role in regenerative processes. Additionally, microenvironmental factors such as oxygen concentration, thrombin activation, angiogenesis, or bioelectric signaling can influence regeneration [42–44]. For instance, during digit regeneration, the oxygen tension can accelerate or attenuate bone mineralization [42]. Cell specification can also be affected by the cellular origin: Analysis of limb regeneration in the axolotl revealed the occurrence of progenitor cells with restricted potential and memory of their tissue origin [45, 46].

The surrounding environment determines associated signaling pathways in regeneration and provides microenvironmental factors, such as oxygen.

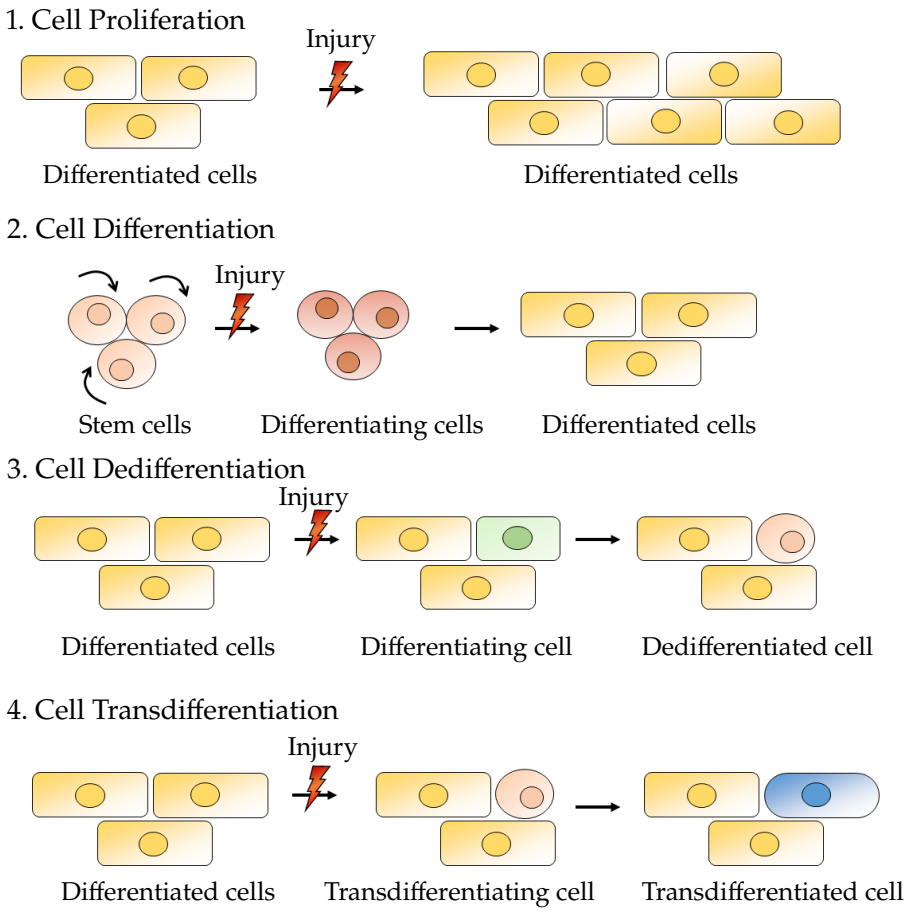


Figure 1: Depending on the regenerating tissue, cells contributing to regeneration can come from different sources. Cell proliferation can produce more differentiated cells during regeneration. Additionally, self-renewing stem cells can contribute to regeneration via differentiation. Dedifferentiation and transdifferentiation, usually via an intermediate step, are further processes involved in regenerating tissue. Parts based on [34].

Mechanics are also connected to regeneration. These can, for instance, involve surface tension and cell attraction and are linked to the cytoskeleton of single cells.

In addition to specific microenvironmental factors and gene expression patterns, mechanics plays a central role in regeneration. Cells can sense the stiffness of the surroundings and mechanical signaling can determine stem cell fate *in vitro* [47, 48]. To understand the role of mechanics, it is important to realize that tissue is an active and viscoelastic material, which can, for instance, undergo fusion events [48]. Mechanical signaling also supports cell sorting [48, 49]. Without surface tension between cells or tissues, a checkerboard pattern forms. A tissue with high surface tension can encapsulate a tissue with lower surface tension. Additionally, without any attractions, two tissues can fully separate [48]. The arrangement of cells is highly linked to the cytoskeleton of individual cells and to the surrounding extracellular matrix. Changes in the cytoskeletal organization or contractility ultimately result in geometric or topological changes on a tissue scale [48]. Furthermore, events like migration, proliferation, or apoptosis are closely linked to cell mechanics: to cell density, substrate properties, and contact inhibition [48, 50–52].

2.2 The interplay of cells in multicellular tissues

The above demonstrates the complexity of the term "regenerative capacity". To address repair and regeneration appropriate models need to be conceived. The investigation of multicellular interactions would, in an ideal case, take place in the native environment and the used techniques should neither interfere with the interactions nor with the studied subject. The used techniques should not cause harm or pain to the studied subject. The fulfillment or approximation of these requirements possesses a high demand for appropriate model systems. Moreover, to understand repair and regeneration, damage needs to be applied. Which laboratory model systems can instruct reparative processes and which techniques would allow us to observe them? The next part will discuss those models from top to bottom, first describing repair and in particular regeneration in *in vivo* models and last repair processes in single cells.

In vivo models of repair and regeneration

As mentioned above, the discovery of the regenerative abilities of the model organism Hydra paved the way to better understand regeneration. Hydra regeneration is based on unipotent epidermal or gastrodermal epithelial stem cells and multipotent interstitial stem cells [53]. It can regenerate a body axis from single aggregates. In a wounded Hydra, interstitial progenitor cells migrate towards the wound [53]. Planarians are model organisms with similar regeneration. A partly chopped planarian worm can also regenerate from each piece back into an animal within two weeks [54]. Wounding activates so-called neoblasts' (adult stem cells) division, which migrate to the wound and form the blastema [53, 54]. In developing Drosophila regeneration after damage also occurs via blastema formation, in adult Drosophila multiple regenerative concepts have been observed [55]. Salamanders can regenerate entire limbs, ocular tissues, parts of the central nervous system, tail, and heart [46]. Limb regeneration in salamander is well understood - it involves blastema formation and reprogramming of cells, which keep their positional memory during regeneration [45, 46]. Zebrafish have a high regenerative capacity and have been studied extensively due to the good experimental accessibility and the huge armamentarium of genetic tools [56].

The most popular *in vivo* regeneration models are Hydra, planarians, Drosophila, salamanders, zebrafish, and mouse. They all show different regeneration competencies. Mammals have the worst ability to regenerate tissue.

The spiny mouse is nowadays a popular mammalian regeneration model. It does not show fibrosis in response to tissue injury and can to a high extend regenerate the ear, spinal cord, kidney, skin, and skeletal muscle [56]. Other mammals are usually regarded as regeneration incompetent, at least not showing unusual/reparative regenerative pathways, which are not linked to normal tissue turnover [56]. However, there are some exceptions. These include liver regeneration, which can be well studied in rodent models and involves a strong regenerative response that is able to restore two-thirds of liver mass after partial hepatectomy in a time course of a few weeks [57, 58]. Additionally, in newborn mice, full cardiac regeneration is possible [59].

In vitro models of repair and regeneration

The cultivation of cells or tissues has enabled seminal insights into the broad cellular interplay during repair and regeneration and into stem cell biology [60]. Transplantation experiments with isolated murine bone marrow cells, which were transplanted into supralethal irradiated mouse spleen and led to colonies of proliferating cells, pointed out that adult tissue contains stem cells - in this case, hematopoietic stem cells [60, 61]. Additionally, the isolation of bone marrow mesenchymal stem cells from guinea pigs has not only allowed us to better understand stem cell-related processes but also paved the way to several clinical trials on mesenchymal stem cells [62, 63]. In 2006, Takahashi and Yamanaka demonstrated the reprogramming of mouse embryonic and adult fibroblasts by the introduction of Oct3/4, Sox2, c-Myc, and Klf-4 as a hallmark in stem cell biology and for future regenerative therapies [64].

While two-dimensional cell culture allows studying cell lines, primary cells, or stem cell-derived cells in mono- or mixed cultures, three-dimensional cell culture can pave the way to better understand cellular interplay in tissue and environmental conditions. In particular, organoids present a powerful culture technique to study regenerative processes. An organoid is defined by a cellular three-dimensional structure, which consists of organ-specific cell types. Organoids are grown from stem cells and self-organize through cell sorting and spatially restricted differentiation [65, 66]. The stem cell source can be either adult stem cells or embryonic or induced pluripotent stem cell lines. Different developmental signals, which are employed via specific culture conditions, enable the derivation of many different organoid types - including stomach, intestinal, lung, thyroid, liver, or brain organoids [65, 66]. Intestinal organoids are currently researched extensively, for instance, to better understand the interplay between crypt with stem cell niche and intestinal epithelium. In the absence of supplementary Wnt ligands in the culture medium, colonic organoids do not maintain colon stem cells. Conditioned medium enables to identify and track LGR5-GFP+ stem cells in colonic organoids [67]. Organoids have been used as *in vitro* disease models, for example, to study intestinal bowel disease [68]. It is also possible to genetically modify organoids via viral transduction, or transfection via lipofection and electroporation [69–71]. Schwank et al. demonstrated the delivery of bacterial artificial chromosomes into organoids [70]. Furthermore, the genetic modification via fluorescence labels allows to follow cells in the organoid. However, this can only be accomplished with close observation and demands high technical requirements - *often light based*.

Organoids are an emerging promising *in vitro* technique to understand disease and regeneration. They resemble the three-dimensional composition of organs on a small scale and are derived from adult or induced pluripotent stem cells.

Next to the cellular origin, specification, and interplay, the understanding of cells on a single-cell level is important to fully understand repair and regeneration. How would a single damaged cell react? Can it restore its function? Would it evoke any signaling on other cells?

2.3 Understanding the single-cell level

The importance to understand the role of single cells in a reparative response becomes clearer with two examples: The first one deals with apoptosis, a central process in biology. Single cells can undergo apoptosis - programmed cell death - based on intra- or extracellular signals. In regeneration, apoptosis is strongly related to the MAPK and the Wnt signaling pathways and linked with proliferation. Apoptosis can be necessary and also sufficient for certain regenerative processes [72]. As a second example, differences in the subcellular structure play a role in regeneration - and regeneration can even reconfigure subcellular structures. For instance, muscle cells have a sarcomeric cytoskeleton, which is responsible for force generation. In cardiomyocyte regeneration in zebrafish hearts, cardiomyocytes can undergo dedifferentiation to start proliferation [30, 73]. This implies that the sarcomeric cytoskeleton is reconfigured [72].

Tissue regeneration is linked to the function of single cells. Therefore, also single-cell damage needs to be understood.

On a single-cell level, repair and remodeling will be used as terms to describe the process of "reaching the original state after damage". How do single cells perform repair and what are the associated responses? To understand this, damage models on a single-cell level, which allow to include both, cellular interplay and intracellular cell-specific changes, need to be existent.

Repairing cell damage

In general, repair of single cells can be considered similar to a regenerative process: After an injury, sealing of the wound occurs, followed by reconstitution and remodeling of the damaged area, which finally restores its normal function [74]. Specific damage to cells can be manifold and based on external physical factors such as heat or radiation, metabolic or chemical changes of the environment, or microbial interactions. However, cell damage also occurs frequently, even during normal tissue homeostasis *in vivo*: Muscle cell contraction can, for example, induce reversible wounding of the cell membrane: Clarke et al. demonstrated, the occurrence of serum albumin in a quarter of rat cardiomyocytes. The albumin entered the cell through reversible wounding of the plasma membrane [75].

Cell damage can be external, for instance, chemical, physical, or microbial or it can occur during normal homeostasis, for example, due to extensive cell contraction.

A well-understood process in cell damage is membrane repair. After membrane puncture, sealing of the wound is proposed to occur via a combination of six different mechanisms: contraction with inward closing of the intact membrane, exocytosis of damaged membrane protrusions, patching of the membrane at the wound borders, internalization of the damaged membrane regions, externalization of the regions, or plugging of membranous compartments of the cytoskeleton [76, 77]. The process is highly dependent on the cell type, cell size, and wound diameter. All membrane repair processes are highly calcium-dependent. Without external calcium, which would flow into the cell upon wounding, no membrane repair can occur [76]. The calcium triggers in an initial resealing

Several pathways to repair membrane damage are existent. They require calcium and rely on a central role of the cellular cytoskeleton.

phase an accumulation of calcium-activated phospholipid-binding proteins within 10 to 45 s at the damaged membrane site. In particular, proteins of the annexin family are recruited to the wound and might orchestrate the membrane resealing [78]. Annexins also contribute to the full repair of the membrane [78]. The membrane is attached to the cytoskeletal actin cortex, which leads to tension forces upon wounding. Furthermore, additional tension forces act at the wound edge. To stabilize the wound and to avoid its increase, the actin cortex must temporally disassemble to release tension [74, 77]. After the initial phase of membrane regeneration, membrane remodeling to restore the native function is necessary. It is typically accompanied by cytoskeletal remodeling at the site of injury, especially the actin cortex needs to get reassembled [77]. Furthermore, lysosomes are exocytosed during cell regeneration and can also fuse with the membrane [79].

Further well-studied types of cell damage include DNA damage or mitochondrial damage. Upon DNA damage, a signaling cascade within the cell is activated. This either leads to damage removal or repair of damage via direct repair, base excision repair, nucleotide excision repair, double-strand break repair, and cross-link repair - or to cell apoptosis [80]. It involves several checkpoints, a halt of cell cycle progression, and beneficial transcriptome changes [80]. Mitochondrial damage is usually connected to chemical exposure, but can also be a secondary event during cell injury [81]. Damage to mitochondria is severe as they are responsible for cellular energy homeostasis and respiration. Furthermore, they are connected to several signaling molecules, for instance, calcium signaling or reactive oxygen species (ROS) [82]. Reactive oxygen species such as singlet oxygen, hydrogen peroxide, superoxides, or hydroxyl radicals are highly reactive. During normal cellular homeostasis, ROS are byproducts of the process of oxidative phosphorylation and can also function as signaling molecules [83]. Negative external stimuli, such as damage by UV light exposure, heat, or irradiation can lead to a significant increase of ROS formation, termed oxidative stress, in the cell. ROS increase can lead to severe damage of mitochondria, if the antioxidant defense system, with enzymes such as superoxide dismutase is overwhelmed [84, 85]. The response might be further accompanied by ROS-induced ROS release, a positive feedback loop of an elevated ROS release upon elevated ROS exposure [86, 87]. ROS can cause a catastrophic permeability of the inner mitochondrial membrane, which gets permeable to all solutes up to 1500 Da [81]. This is linked to mitochondrial swelling, depolarization of membrane potential, and inhibition of oxidative phosphorylation [81]. If the damage is beyond repair it can result in loss of a mitochondrion or even cell loss. ROS can also lead to mutations of mitochondrial or nuclear DNA and are connected to carcinogenesis [88].

Damage to mitochondria is often associated with the formation of reactive oxygen species such as singlet oxygen or superoxides.

If a cell is not repaired and dies via necrosis or apoptosis, damaged cells might be replaced. In this context, it is important to note that many findings on single-cell repair are based on *in vitro* cell culture without consideration of environmental cells [74]. In multicellular wounding and regeneration, migration plays a central role, as detailed above. Migration is based on forward protrusions and rearward retraction forces exerted by single cells. Therefore, the cytoskeleton of individual cells as well as cell-to-cell adhesion systems are highly involved. In the repair of wounds, the central cytoskeletal components actin and non-muscle myosin II lead to wound closure [74]. Abreu-Blanc et al. point out that on all scales, in single-cell, tissue repair, cytokinesis, or morphogenesis similar cytoskeletal elements such as actin, Rho family small GTPases, or E-Cadherins are utilized, underlying again a strong connection between single-cell and multicellular repair and regeneration [74]. This reflects the ability of the cell to adapt these elements and pathways for multiple biological functions.

2.4 Optical visualization of repair and regeneration

To follow multicellular processes and understand single cells, we are interested in several measures. A full perspective would include knowing, which cells occupy a defined position at a defined time. Furthermore, we would ask for each cells' genome, transcriptome, proteome, and metabolome. Today, single-cell sequencing techniques ("omics") allow high-throughput cell analysis with large amounts of generated data [89, 90]. However, these cannot stand alone and still require *in situ* visualization of the cells and their molecular characteristics - *seeing is believing*.

Imaging of cells *in vitro* , *in situ*, and *in vivo*

Today, the observation of cells in a two-dimensional cell monolayer *in vitro* culture is an easily achievable task using light microscopy. Moreover, already based on the discoveries of Ernst Abbe on the diffraction limit, it is evident that some subcellular structures are within the resolution limit of a light microscope. Super-resolution microscopy techniques are even able to break this limit and reach nano-resolutions [19]. Most of the observations in cell biology require sufficient contrast. Due to the cell composition and high water content, which provide its transparency, the amplitude of light is (almost) not changed if it travels through the cells. Only phase changes of the light wave occur, which can be visualized by phase-contrast microscopy or digital holographic methods [91], providing some contrast. However, even the latter techniques cannot reach as high contrast as fluorescence staining. Consequently, cell biology is usually associated with chemical fluorescence staining or the expression of fluorescent proteins.

Many microscopy techniques have evolved and allow imaging over various scales. However, most of these require additional contrast, which is only reached via fluorescence imaging.

If living cells need to be stained via a chemical dye, commonly membrane permeability of the dye is required. A high number of dyes for cell staining is nowadays available and due to their different chemical composition, various

sub-cellular structures can be targeted and visualized. Antibody-based staining typically requires fixation of cells and subsequent permeabilization as antibodies cannot penetrate the cell membrane. Antibodies that are sensitive to surface antigens can be used for the staining of living cells and are sufficiently stable for even long-term experiments [92]. The use of single-domain antibodies might even help to facilitate this strategy.

The expression of fluorescent proteins is also well-achievable in two-dimensional cell culture. The required delivery of nucleic acids for the expression of the fluorescent protein can be achieved via transfection or transduction of cells. Common transfection techniques such as lipid-based transfection, calcium phosphate precipitation, or electroporation usually yield sufficient efficiency and high cell viability. In difficult to transfect cells, transduction, for instance, via a lentiviral approach, might provide better efficiencies.

Specific labeling of subcellular structures can be achieved via fluorescent fusion proteins. The use of fluorescently labeled and endogenously expressed single-domain antibodies has also been demonstrated for this purpose [93]. To allow cell-specific expression, promoter- or enhancer-driven fluorescent protein expression can be applied. However, this approach is limited by the promoter activity, which needs to provide sufficient basal expression to overcome the autofluorescence [56]. Additionally, a promoter-driven approach might not fully correspond to a given gene expression pattern [56]. As an alternative, genome editing methods such as CRISPR/Cas9 or TALEN would allow a highly specific and efficient targeted genomic integration of a fluorescent protein [94, 95].

Fluorescent proteins can be tagged to specific proteins or expressed in specific cells via promoter or genome editing-based approaches.

The described use of fluorescent proteins is also possible in three-dimensional cell culture or model organisms. In the latter case, usually, fluorescent reporter lines are generated via various techniques. These can also be combined with site-specific recombinase technologies, such as Cre/Lox recombination, to allow further options for cell-specific labeling [56]. Additionally, photomodulatable proteins, which can be turned on or spectrally converted by light can be used for lineage tracing [56].

Imaging in three-dimensional settings, especially in larger model organisms, is challenging. One option for imaging would be the excision of the tissue of interest and the preparation of histological sections. However, this strategy poses a huge problem: It is highly limited to achieving temporally-well resolved data. Moreover, all sections have to be realigned in a three-dimensional dataset and axial spatial resolution is limited by the section thickness. This highly limits the applicability of histology in studying regeneration and diseases.

When stem cells or complex cellular pathways are involved, nearly continuous observation is desirable. If this is not achieved, important events such as cell death or migration can be missed (see Figure 2) [96, 97]. Consequently, imaging techniques, which allow access to the tissue of interest are required. Furthermore, these need to leave the tissue unaffected, such

In repair and regeneration, stem cells or complex cellular pathways are involved, which demands a nearly continuous observation. If this cannot be achieved, important cellular events might be missed.

that imaging associated damage artifacts, for example, phototoxicity, need to be minimized. While this can be realized for *in vitro* tissue the *in vivo* situation is challenging.

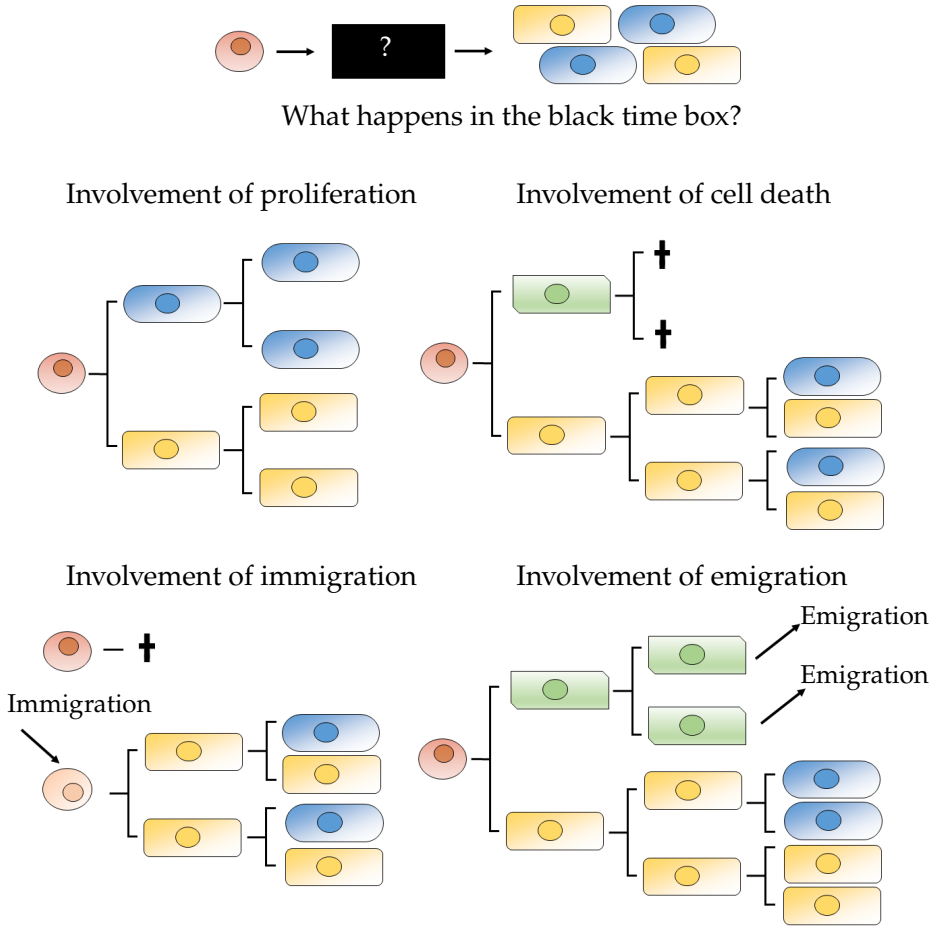


Figure 2: Understanding multicellular interplay, in particular, in the case of regeneration or stem cell biology, requires close observation. The temporal and spatial resolution needs to be sufficiently high to observe processes as proliferation, cell death, immigration, or emigration from specific tissue regions. Based on [96, 97].

Imaging techniques for cellular three dimensional imaging

Today, a variety of imaging techniques for three dimensional cellular imaging exists (see Figure 3). These techniques overcome the problem of widefield epi-fluorescence microscopy that out-of-focus light leads to a blurred image with specimen larger than approximately 10 µm. In confocal microscopy, out-of-focus light is rejected from reaching the detector, based on initial ideas patented by Marvin Minsky in 1955 [98]. Today, typically, laser scanning confocal microscopes are used in cell biology research. These use a visible laser as a point source, scanning mirrors to steer the beam over the sample, and a pinhole to reject out-of-focus light from reaching the detector. If a non-resonant galvo-scanner system is used, images can be gathered with frame rates of about 2 Hz for a size of 512x512 pixels. This speed is sufficient for many cell biological applications. However,

certain conditions require higher speed. For instance, calcium oscillations in neurons occur at a timescale of milliseconds. Consequently, calcium imaging would require much higher image acquisition rates. Using a resonant galvo-scanner system can increase imaging speed up to about 30 Hz [99, 100]. Another approach is to use multiple beam illumination and detection. This is realized in spinning disk confocal microscopy, which is in particular suitable for specimen with sizes of less than a few ten micrometers. It utilizes a rotating disk with pinholes, which are spaced sufficiently apart and therefore creates multi-beam sample acquisition with high speed [101]. However, thick specimen lead to cross-talk between the pinholes and typical spinning-disk microscopes use fixed pinhole sizes, limiting the choice of the numerical aperture of the objective.

As an alternative to confocal microscopes, light sheet microscopy, also called selective plane Illumination microscopy (SPIM) emerged [102, 103]. It uses a thin light sheet that is created by a cylindrical lens and allows two-dimensional illumination of the sample through the objective. In a typical light field setup, another objective is placed orthogonal to the illumination objective and serves to detect the emission light. The specimen is moved and rotated through the image plane to gather three dimensional information. Many modifications of light field setups have been developed, for instance, to allow detection similar to an inverse microscope [104, 105]. By using Bessel beams or optical lattices, which are periodic interference patterns, resolution can be enhanced significantly in light sheet microscopy and even live cell imaging with axial resolutions less than 100 nm is possible [106–108]. The fast acquisition time, which is attributed to the two-dimensional illumination, of three dimensional data for a single specimen makes light field microscopy an attractive technique, for example, to analyze Zebrafish or Drosophila development [105]. However, this also puts limitations on the usable sample geometries and sample mounting.

Compared to a confocal microscope, a light sheet microscope enables faster sample acquisition times as a thin light sheet is used for illumination.

The yet described techniques used linear excitation of the sample with visible light. Consequently, a usual confocal microscope has a limited penetration depth, depending on the sample, which is usually in the range of 100-200 µm. If a laser scanning microscope is used with nonlinear excitation, this is referred to as multiphoton or two-photon microscopy. The combination of other microscopy techniques, such as light sheet with multiphoton excitation is also possible [110]. Laser scanning multiphoton microscopy was initially developed by Denk et al. in 1990 but its foundation is already based on seminar theoretical work on multiphoton excitation by Maria Goeppert-Mayer in 1931 [14, 111]. As multiphoton excitation has a very low probability, multiphoton microscopy not only requires a spatially focused laser but additionally a concentration of the excitation intensity in time, using ultrashort pulsed laser systems. This leads to the quasi-simultaneous absorption of typically two photons using femtosecond laser oscillator systems in the near infrared wavelength regime. Due to the low probability, excitation is intrinsically limited to the focal volume. Because near infrared wavelengths are used and endogenous absorption is low in

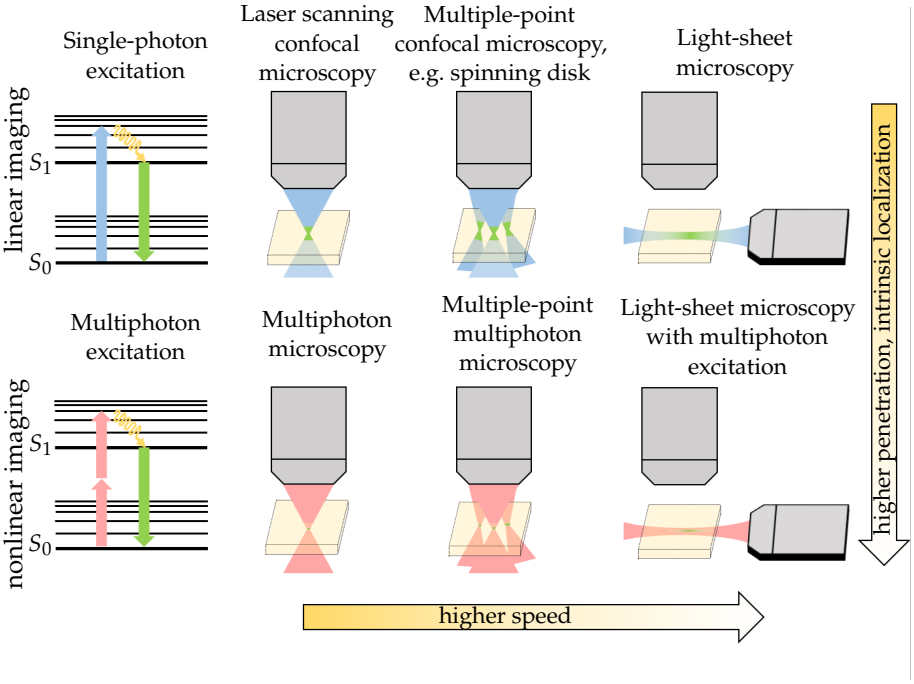


Figure 3: Linear and nonlinear imaging techniques. The penetration depth and intrinsic localization favor the use of multiphoton microscopy. Based on [109].

this wavelength regime, a high penetration depth down to about 500 µm can be realized using standard multiphoton microscopes.

Combined with the high-resolution, the ability to realize optical sectioning, and the possibility to image highly scattering tissue, multiphoton microscopy is outstanding among the light microscopy techniques and consequently several developments tried to improve multiphoton microscopy [112, 113]. A particular interest in multiphoton microscopy is to improve acquisition speed, as it is often used in neurobiology applications. For example, Grewe et al. developed a resonant scanner system and implemented an electrically tunable foucssing lens for fast focus shift within 15 ms. Thereby, two axially separated image planes could be scanned rapidly [114]. Even faster acquisition was shown by Zhang et al. who created a multiphoton microscope with 400 illumination beams through application of a microlens array and a triangle-wave laser-scanning pattern. This allowed to sample areas up to 211,000 µm^2 with 1 kHz rate [115].

Multiphoton microscopy is an outstanding imaging techniques as it allows deep tissue imaging, optical sectioning, to image highly scattering tissue, and high-resolution.

Another improvement often applied in multiphoton microscopy is remote focusing that avoids moving specimen or focusing objective. Instead a replication of the object space is imaged via a second objective and a fast moveable mirror [116]. Scanning of a large field of view with as many cells as possible is also of high interest in multiphoton microscopy. Sofroniew et al. have developed a two-photon random access mesoscope, which could scan an area with a diameter of 5 mm. They combined multiple laser scanners in series and remote focusing to reach diffraction limited performance [117]. Another development in multiphoton microscopy was the use of scan-free image acquisition via spatiotemporal focussing [118].

This approach requires high pulse energies from an femtosecond amplifier laser systems and a diffraction grating produce a pulse, which serves to spatially separate the pulse spectrum such that its frequency components have constructive interference in the focal plane. This provides the required intensity for multiphoton excitation, which can be detected by an EMCCD camera [118]. This widefield approach can consequently reach high frame rates. While multiphoton microscopy usually corresponds to two-photon excitation microscopy, also three-photon microscopic approaches have been developed. Hell et al. demonstrated the feasibility of this approach in 1996 [119]. In 2013, Horton et al. showed three-photon microscopy in the intact mouse brain at the excitation window of 1700 nm [120]. Imaging in the second near infrared imaging window from 1100 to 1350 nm and in the third near infrared window from 1600 to 1870 nm [121] can allow deeper tissue imaging. Additionally, three-photon microscopy is significantly improved in its localization compared to two-photon excitation [120].

Improvements in multiphoton microscopy include imaging large fields of view (mesoscopic imaging), fast-scanning modalities, remote foccusing, or the use of three-photon excited fluorescence in the second and third near infrared wavelength window.

Accessing the tissue of interest

A powerful tool for studying cells *in vivo* in a living organism is intravital imaging [122]. It can be used with wide-field fluorescence microscopy, laser scanning (confocal) microscopy, or multiphoton microscopy. As described above, the latter is the most powerful technique and can be applied for imaging at single-cell resolution in various organ systems. Therefore, since its first demonstration for intravital brain imaging [14], it has found many applications. For instance, multiphoton microscopy of the gut has been performed *ex-* and *in vivo*. Furthermore, the pathology of dextran sulfate sodium (DSS) colitis in mice and associated irregularities of the crypt have been investigated by Morimoto et al. with intravital multiphoton microscopy [123]. Another application is the visualization of infections [124]. Using multiphoton microscopy, Millet et al. studied Vibrio cholera infection, with fluorescently labeled bacteria, in the small intestine [125].

The accessibility of the tissue of interest is still a problem in intravital imaging. In small model organisms such as Caenorhabditis elegans or developing zebrafish embryos, this problem is not so prominent. Sufficient transparency allows a high penetration depth and strategies for immobilization of the specimen can be trouble-free realized. With new imaging modalities such as light-sheet microscopy and special specimen mounting, even long-term imaging over several days is possible [126]. In rodents, the situation is more challenging. A common approach is to surgically expose the tissue of interest, while the animal is anesthetized and analgized. This is usually associated with sacrificing the animal after imaging. As an example, the liver can be well imaged and studied using surgical exposure via laparotomy and static positioning [127]. To overcome the limitations on imaging larger specimens, three different strategies have evolved: imaging windows, fiber or grin-lens based imaging approaches, and miniature microscopes (see Figure 4).

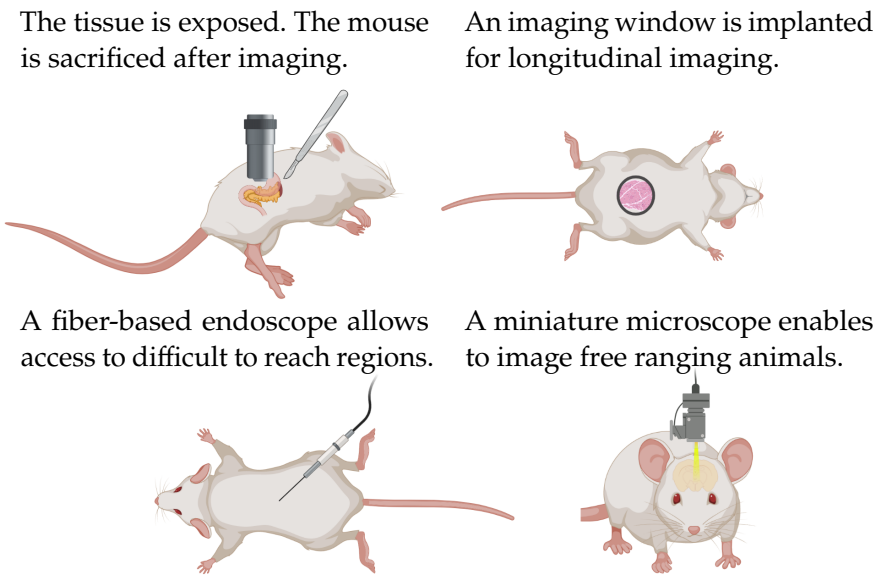


Figure 4: Imaging modalities, which can be used for *in vivo* imaging of larger specimens, such as mice. The imaging window, endoscope, and miniature microscope allow longitudinal imaging. Created with BioRender.com.

Already in 1924, Sandison et al. demonstrated the first use of a transparent chamber for microscopic imaging of a rabbit ear in a period of a week to two months [128]. This method was later adapted to the mouse via the dorsal skin-fold chamber [129]. In this chamber, thin layers of tissue are made accessible to microscopic observation. The use of such windows has been extended to other mouse or rat organs. A brain window can be fixed to the skull after a craniotomy and allows imaging of deep cortex regions without brain inflammation for months (chronic brain window) [130, 131]. This technique even allows to image awake animals, for instance, to record calcium activity within the brain [132]. An important achievement in gastrointestinal imaging was the realization of an abdominal imaging window by Ritsma et al. [133–135]. It allowed studying the steps of liver metastasis formation in a time period of two weeks [133]. Furthermore, it has been applied to study LGR5+ stem cells in the small intestine and revealed the dynamic interaction and transfer of central and border stem cells [135]. Consequently, it can fulfill the demands required to understand stem cell-driven regeneration as tracking is possible on a single-cell level with a high temporal resolution, only limited by the possibility of successive imaging sessions.

Imaging windows, consisting of a glass coverslip, which is mounted or glued to the tissue of interest, allow long-term tissue access and longitudinal imaging.

Another option to access difficult-to-reach tissue is the application of optical fibers in microscopy. Broadly, fiber optics can be used to deliver light, detect light, or for simultaneous delivery and detection. Fiber-based imaging can be reached in combination with wide-field illumination and camera-based detection or in combination with laser scanning systems. In the latter case, two configurations are possible: the scanning system can be placed at the distal end, which is facing the tissue, or at the proximal fiber end. Several scanning mechanisms at the distal end have

been demonstrated, for instance, vibrating the fiber tip or scanning with micrometer mirrors [136, 137]. Scanning of the proximal end is usually performed in combination with coherent fiber bundles. They produce a replicate of the distal end image at the proximal end of the bundle. They are composed of hundreds to thousands of optical fibers and are in the range of a few hundred micrometers to some millimeters. Imaging fiber bundles have been applied in wide-field fiber-based microscopy and in combination with laser-scanning systems [136, 138–143]. For instance, the application of a fiber bundle confocal microscope was demonstrated for colonoscopy in mice [144]. It allowed visualizing yellow fluorescent protein (YFP) expressing epithelial cells in a transgenic mouse and imaging of the vasculature after teil vein injection of fluorescent dextran [144]. Additionally, it holds great promise for clinical exploration, for example, to image the airways via insertion of the fiber bundle through the working channel of a bronchoscope [145].

Fiber-based microscopy often uses fiber bundles, which are small in diameter and can be combined with wide-field illumination and detection or with laser scanning systems.

Animal-mounted miniature microscopes can be combined with fiber-based approaches or work as independent systems [146–148]. Miniature microscopes allow observing cell dynamics in awake, free-ranging animals. This demands the development of devices, which can be carried around by the animal. In particular, head-mounted microscopes have achieved considerable attention in the neurobiology field. As an early example, Rector et al. combined a charged coupled device (CCD) chip camera, a fiber bundle, and light-emitting diodes in a miniature microscope that was placed in either neocortex or dorsal hippocampus of cats [149, 150]. It enabled them to record neural activity, however, not on a cellular level. Helmchen et al. built a miniature fiberscope, which was head-mounted to enable imaging of the rat cortex. They used a single-mode optical fiber to deliver pre-shaped laser pulses to a small scanning fiber scope consisting of scan mirrors, objective, and a photomultiplier tube. This allowed them to investigate dendritic calcium dynamics in freely moving animals [146]. An epi-fluorescence-based approach was demonstrated by Flusberg et al. The microscope optics mounted to the mouse achieved a weight as low as 1.1 g. A fiber imaging bundle served for illumination and collection of light to a distal placed EMCCD camera [148]. Newer developments also include the use of complementary metal-oxide-semiconductor (CMOS) camera sensors in fully-integrated miniature microscopes, which also contain excitation sources and optics [147]. In these cases, only cables for data acquisition and power supply are necessary. Recently, the first wireless miniature microscopes were developed [151].

Miniature microscopes, with weights as low as 1.1 g, allow investigating cell dynamics in free-ranging animals.

Image analysis of cell dynamics

Visualization is key to understanding repair and regeneration. As detailed above, several imaging modalities are existing to address this issue. Nevertheless, the visualization of reparative and regenerative processes not only requires successful imaging but is also ultimately linked to the careful

interpretation and presentation of the imaging data. This can pose a challenge with data of modern microscopes. Images can be acquired in high digital resolution, which results in an increasing number of images in large data files. Often, three-dimensional imaging data is recorded, for example, via confocal or multiphoton microscopy. Moreover, several fluorescent channels are usually needed to visualize complex processes. Furthermore, a high temporal resolution is demanded, if cellular or subcellular dynamics are of interest. This results in n-dimensional datasets, which have to be presented and analyzed on the computer. It can also necessitate trade-offs between resolution, speed, noise, and imaging depth.

Another challenge is image registration: For example, different time points in image data have to be registered with each other [152]. In intravital microscopy, this can be a difficult task. Motion artifacts such as heartbeat, breathing, or peristalsis can easily lead to image degradation [153]. A hardware adaption of the imaging method to these artifacts would require good a priori knowledge of their periodicity and morphology. Novel visualization and analysis tools can allow raw data acquisition and digital compensation of motion artifacts [154].

Several software tools for manual image analysis are available and automated tools for high-throughput analysis are in development [155, 156]. Additionally, deep learning has a significant impact on the developments in microscopy data visualization and analysis. Deep learning uses multilayered artificial neural networks. These are trained on annotated image data. Weigert et al. showed the application of a convolutional neural network, based on the U-Net architecture, for the restoration of fluorescence images [157, 158]. Images with significant noise could be improved via the application of the network. While this required separate training data, also other networks have been created, which allow noise removal based on direct training with the data acquired during an experiment [159]. Additionally, deep learning also benefits several other directions in image analysis, such as segmentation or *in silico* labeling of cells [158, 160].

Deep learning has a significant impact on the developments in microscopy data visualization and analysis. It uses multilayered artificial neural networks and can, for instance, serve to reduce noise, to provide in-silico labeling, or segmentation.

2.5 Manipulation of cells *in vitro* and *in vivo*

For accessing reparative dynamics, it is also important how cells and tissue can be manipulated. When biologists talk about manipulating cells or model systems, they usually refer to genetic manipulation. In 1974, the first transgenic mouse was created via the insertion of Simian Virus 40 DNA into an early-stage mouse embryo [161]. To obtain transgenic mice, several techniques exist, including pronuclear injection or genetic modification of embryonic stem cells, which are injected in mice blastocysts [162].

Today, genome editing via engineered nucleases is the method of choice for removing genes ("knock-out"), replacing genes ("knock-in"), or producing mutations [163, 164]. The principal idea of genome editing is to induce double-strand breaks via the nucleases and use the cellular repair processes

non-homologous end-joining (NHEJ) or homology-directed repair (HDR) to produce the desired genetic modification [163, 164]. Genetically modified cells can lack genes, are reduced in the expression of chosen genes, overexpress specific genes, express novel genes, or express genes with mutations. A genetic modification can also be conditional, which means that it is activated under defined external conditions [165–167]. Such a condition can be a specific time point in development or the administration of a chemical, for example, the selective estrogen receptor modulator tamoxifen in a tamoxifen-dependent Cre recombinase expressing mouse [166, 167]. In combination with loci containing loxP sites, the latter approach can be applied for the temporally and spatially confined generation of site specific mutations [167]. Principles of genetic manipulation usually come into play, when ways to enhance regeneration are discussed.

Next to genetic manipulation, several other methods of manipulating cells or tissue exist. Especially external stimuli can serve to modify cells. These include, for example, chemical treatment of cells. *In vitro*, chemicals can be added to cells. In stem cell biology, for example, small molecules are tested and applied as these can significantly help to control the state, fate and function of a (stem) cell [168]. However, chemical treatment is highly unprecise and allows a very limited targeting of defined cells. Physical methods can be more specific. Electrical manipulation can be applied via spatially-confined electrodes. An example of electrical manipulation is electroporation, which uses an electric field to induce a rapid of rapid membrane structural rearrangement with pore formation [169]. This can deliver molecules into cells in cell transfection. Mechanical manipulation can, for instance, enable inserting material into cells via microinjection, which uses a glass needle to inject femtoliter volumes into cells. Cryoablation or cryoinjury is another manipulation technique that kills cells by rapid cooling. It uses a cryo needle and either directly injures cells or changes the microenvironment for cells further away from the cryoneedle, such that their survival is limited [170]. This method is used in connection to cancer treatment in clinics, but can also be applied on an *in vitro* scale. Voges et al. have developed a human cardiac organoid injury model, which used cryoinjury in stem cell-derived organoids to analyze regeneration. They used a piece of dry-ice to perform injury [171]. An atomic force microscope with a modified tip can also be used for cell manipulation. Obataya et al. developed such a system with a needle of 200 to 300 nm diameter and 6 to 8 µm in length. This needle could be precisely inserted in the HEK293 cells and resulted in minimal deformation of the cells [172]. Also magnetic particles and external fields can be used for physical cell manipulation. Liu et al. showed a modulation of cell orientation and migration using cell-internalized iron oxide particles in an external field [173].

Non-genetic cell manipulation can be realized via chemical treatment or physical methods, including electrical manipulation, cryoinjury, or modified atomic force microscopy approaches.

All of the latter approaches require to add something to the cells or to use a probe that touches the cell and can also barely reach single cell precision. Therefore, another method for cell manipulation is highly desireable. *The application of light for cell manipulation is such a powerful method.*

Light can be another powerful tool to manipulate biological systems

Light-based cell manipulation: Cell surgery

Already in 1887 Thomas P. Downes and Arthus Blunt discovered an inhibitory effect of ultraviolet (UV) light on bacterial growth and found a dependence of the strength of inhibition on exposure time, intensity, and wavelength [174–176]. In 1905, Ernst Hertel quantified the amount of UV light, which is necessary for bacterial cell death [177–179]. He also performed experiments using different wavelengths and tried to kill cells in the developing sea urchin by illuminating the blastomere in the two- to four-cell stage [179, 180]. These experiments inspired Sergei S. Tschachotin in 1912 to develop the so-called "mikroskopische Strahlenstichmethode" [180]. His idea was to use a fine beam of UV light to illuminate certain cell regions, in particular the cell nucleus. This was the first application of focused light for cell manipulation [180]. After this first attempt by Tschachotin, several studies have used extended light sources with partial focusing for cell manipulation [181, 182]. In 1960, the first laser was developed by Theodore Maiman, which pushed the application of focused light for cell manipulation to a new level [183]. A laser can be focused to a diffraction-limited spot, in contrast to an extended light source. Consequently, for the first time, irradiation of small subcellular structures in the range of several hundred nanometers was possible after the first laser was available.

The "mikroskopische Strahlenstichmethode" was the first demonstration of precise manipulation of cells using light.

In 1962, Bessis et al. employed for the first time a ruby laser, which was coupled into a microscope, for precise cell manipulation [184, 185]. Shortly after, a ruby laser with 500 µs irradiation time was applied to precisely manipulate only the area of interest in algae without irreversible damage to the surrounding area [186]. Additionally, the application of other laser types coupled into microscopes, such as a monochromatic continuous-wave helium-neon laser, was evaluated successfully for the manipulation of chicken embryos and HeLa cells [187]. Starting in the 1970s, Berns et al. reported in several seminal publications the use of argon laser micro-irradiation for cell manipulation. First studies focused on the manipulation of chromosomes and mitochondria and several other open questions in biology were subsequently targeted [188–191]. While these studies were very valuable for many biological problems, the open question remaining was if there is still potential to improve laser microsurgery.

This question was answered in 1999 when Koenig et al. showed the application of femtosecond laser systems for intracellular nanosurgery [192]. This system enabled to confine the deposited laser energy to the focal volume, mainly due to nonlinear photo ionization. The nonlinear processes also imply a lower dependence on the absorption cross-section of the target material [193]. As femtosecond laser systems usually operate at near-infrared wavelengths, a high penetration depth into the tissue can be realized. Based on these aspects, the precision of laser manipulation could be enhanced two- to threefold, compared to continuous wave irradiation [193].

Femtosecond laser systems in the near-infrared wavelength regime enable precise manipulation of biological systems and provide high penetration depths.

To understand the mechanisms of femtosecond laser nanosurgery, Vogel et al. have developed a model, which considers the formation of an electron

plasma in water due to the application of a tightly focused femtosecond laser pulse [193]. In a first step, this evokes photo ionization of water molecules. This leads to the production of free electrons, so called seed electrons (see Figure 5). The photo ionization process can be either caused by multiphoton ionization or tunnel ionization, which has a stronger impact for high field strength [193]. In the near-infrared window around 800 nm, multiphoton ionization outweighs tunnel ionization. However, as the probability of multiphoton ionization decreases with increasing wavelength, while the probability of tunnel ionization stays constant, their ratio of contribution to seed electron formation is wavelength dependent [194]. At 2000 nm tunnel ionization would dominate multiphoton ionization [194]. The seed electrons can be further accelerated by Inverse Bremsstrahlung, which can generate further free electrons through impact ionization. This causes an avalanche growth of free electrons [193]. If the plasma density of free electrons is sufficiently high, optical breakdown occurs.

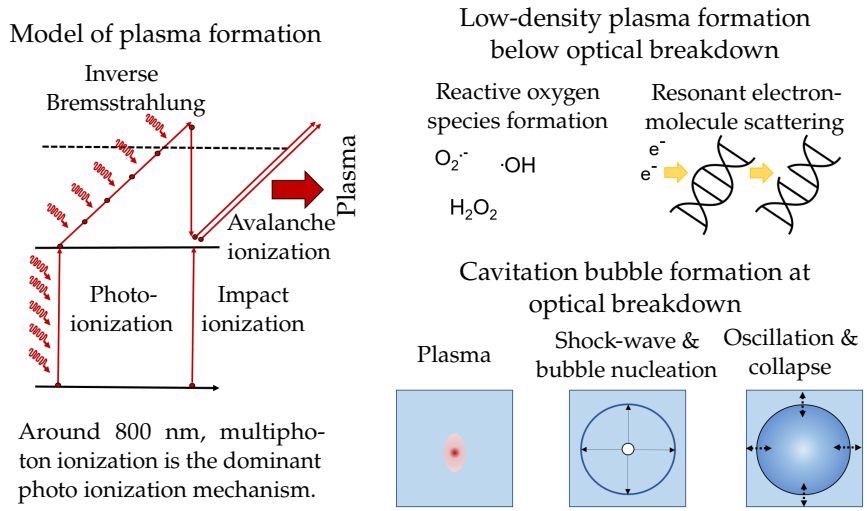


Figure 5: Schematic depiction of mechanisms of femtosecond laser nanosurgery (in water) based on [193]. Photo ionization leads to the formation of free electrons. Inverse Bremsstrahlung accelerates these seed electrons such that they collide with atoms and the impact leads to further free electrons, ultimately resulting in avalanche ionization. This leads to plasma formation. The plasma can be a low-density plasma or lead to cavitation bubble formation, depending on the irradiance.

Based on this, two regimes of manipulation have been described and used [193]. In the so-called low-density plasma regime below optical breakdown threshold, photo ionization of water- and biomolecules yields free electrons, which can lead to ROS formation or direct changes of biomolecules via resonant electron-molecule scattering [193]. At a certain irradiance threshold, an optical breakdown occurs. The optical breakdown is connected to the formation of a shock wave and the nucleation of a cavitation bubble, which can allow cell manipulation in the second regime [193]. In the near infrared regime around 800 nm, multiphoton ionization is the main process driving low-density plasma formation. Therefore, the electron density in the low-density plasma can be well-tuned below the optical breakdown as it is mainly dependent on the

irradiance [193]. The bubble threshold is determined by the contribution of avalanche ionization and the ratio of avalanche ionization to photo ionization increases strongly for higher wavelengths, as the multiphoton ionization rate decreases stepwise with each necessary photon, while the rate of avalanche ionization increases [194]. A typical laser setup operating for nanosurgery in the low-density plasma is a laser oscillator at 80 MHz, while above the optical breakdown a laser amplifier system at single pulse or repetition rates around 1 kHz is used [193].

The first demonstrations of the power of femtosecond laser nanosurgery included the nanodissection of chromosomes and the ablation of cytoskeletal elements and mitochondria (see Figure 6) [195, 196]. Pioneering work on the application of femtosecond laser manipulation for biological problems comprised the demonstration of the viscoelastic properties of actin stress fibers, studies on nerve regeneration after precise axon damage in Caenorhabditis elegans, and the demonstration of Stroke formation after microvessel targeting in the rodent brain [197–199]. Additionally, femtosecond laser transfection, a technique, which employs laser pulses to reversibly porate the cell membrane was demonstrated and extended by several groups [200, 201].

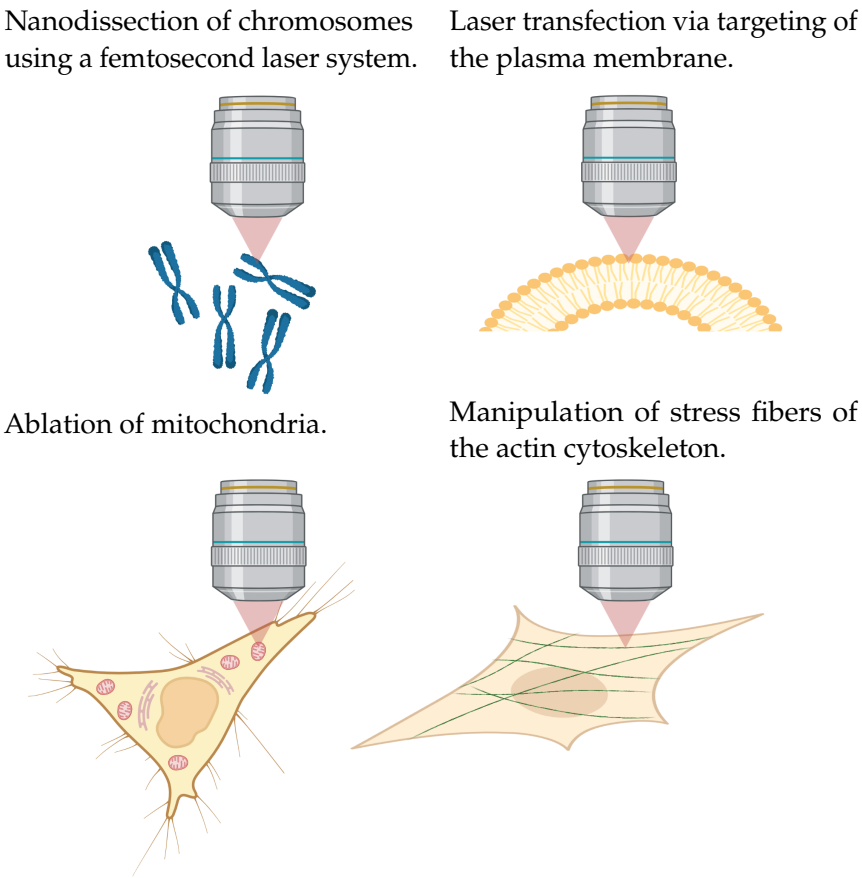


Figure 6: Applications of femtosecond laser nanosurgery. For example, nanodissection of chromosomes, plasma membrane poration, ablation of mitochondria, and manipulation of the cytoskeleton have been demonstrated. Created with BioRender.com.

Further applications of light-based cell manipulation

Other applications of light in biological manipulation include the use of optical tweezers and optogenetics. Optical tweezers can trap and move objects, while optogenetics uses light to trigger specific cellular events in genetically modified cells with photosensitive proteins.

Optical tweezers were developed by Ahskin et al., who also demonstrated their first application in cell trapping [202–204]. The basic idea is to use the scattering force, which describes the transfer of momentum from the photons to the tweezed particles, and the gradient force, which is produced by the gradient of intensity along the particle's axis [205]. Various optical configurations for optical tweezers exist and have found widespread use in biology as they can deliver forces of the order of piconewtons [206–208]. A striking example is the sorting of cells in fluids, which allows them to be moved over large distances [209]. *In vivo*, optical trapping is difficult as light is scattered and loses power with penetration of the tissue. Nevertheless, some pioneering studies have demonstrated the trapping of red blood cells in subdermal mouse capillaries and the trapping of the large otoliths in larval zebrafish [210, 211].

Optogenetics can be broadly defined as the control of cellular activity by light. One application of optogenetics is the light-based triggering of photo-sensitive ion channels [212–214]. These include in particular channelrhodopsins, but also other rhodopsins such as halorhodopsin or archaerhodopsin [214]. The cells need to be genetically modified to express these channels. Optogenetic-based triggering of photo-sensitive channels has found numerous applications in the neurobiology field to trigger neurons with millisecond temporal resolution, even in freely moving animals as well as in the cardiac field, for instance, to pace the cardiomyocytes and heart muscle in mice [212, 215, 216].

In another application of optogenetics, light is used to control other photo-sensitive proteins. This includes, for example, proteins from the light-oxygen-voltage (LOV) or cryptochrome families. Kennedy et al. have used blue-light triggerable protein modules from Arabidopsis thaliana Cryptochrome 2 (CRY2) and CIB1 to create a split Cre recombinase, which components are functionally fused upon illumination [217]. Such results unveil a great potential: optogenetics can be used to control intracellular processes and gene expression, which makes it a versatile tool in developmental and regenerative biology [218]. For example, the light-based reversible activation or inactivation of photoactivatable Rac in the Drosophila ovary enabled to induce forward or backward motion of the border cells [219]. Furthermore, in the Drosophila embryo an optogenetic CRY2-CIB1 protein dimerization system was used to control the levels of phosphatidylinositol-4,5 bisphosphate, which is involved in actin polymerization and cell contractility. This allowed to modulate apical constriction and using two-photon illumination a high spatial precision was reached [220].

Optogenetics can be applied to activate photosensitive membrane channels. Additionally, other photosensitive proteins have been derived, which can even allow to control gene expression.

2.6 Applying engineering principles and optics in regenerative medicine

A short definition of the field of regenerative medicine reads "Regenerative medicine replaces or regenerates human cells, tissue or organs, to restore or establish normal function" [221]. It is an interdisciplinary field, which involves different aspects from medicine, life sciences, and engineering. It has attracted governmental awareness and several regenerative medicine products have reached the market or the preclinical stage and clinical testing [221, 222]. It has a strong motivation from the limited donor supply in case of organ loss or failure [223]. With the central ideas of tissue engineering and artificial organ supply, it includes the fabrication of grafts or mimics and techniques for their integration [222]. Moreover, regenerative medicine also deals with the enhancement of the host's regenerative capacity by alteration of the tissue environment, cell injections, or immune modulation [222].

Stem cells for tissue engineering

In the center of regenerative medicine are stem cells. Due to the differentiation potential of embryonic stem cells or induced pluripotent stem cells and the plasticity of adult stem cells, these make ideal candidates for creating bio-artificial tissue, for cell-based therapies, or for being targeted in the patient to stimulate regeneration [28]. In *in vitro* directed differentiation, stem cells are pushed to the desired cell type via mimicry of steps that occur during embryonic development, including the application of growth factors or small molecules, co-culture systems, or in some cases, spontaneous differentiation [224]. Reprogramming (trans-differentiation) offers an alternative. It applies factors, which drive trans-differentiation of cells available from the patient, such as fibroblasts, to the therapeutically relevant cell type [224]. The selection of reprogramming factors mainly includes transcription factors, which are key to determining cellular identity [224]. However, other options such as micro RNAs can also yield successful cell reprogramming [225–227]. Many demonstrations such as reprogramming of mouse and human fibroblasts to neurons, cardiomyocytes, or hepatocytes show the high potential of this approach [227–231].

The generated cells are usually applied as building blocks for tissue engineering, which involves their assembly in a tissue scaffold, to allow cell aggregation in controlled environments and microarchitectures [223, 232]. Both top-down and bottom-up strategies are applied in tissue engineering. In top-down approaches, the cells are seeded in a scaffold and stimulated by growth factors or external stimuli, while bottom-up approaches use small tissue modules which are assembled together because many tissues are comprised of repeating function units [232]. Tissue engineering is posed with several challenges, the materials have to be mechanically robust and show functional microvasculature [232]. Additionally, for

Stem cells are central to regenerative medicine. They are used to create building blocks, which are assembled in tissue engineering.

the translation of regenerative medicine products into clinical settings, large-scale manufacturing, and appropriate testing methods need to be available [232].

Enabling techniques in regenerative medicine

To reach the goals of tissue engineering, enabling technologies are necessary. These can, for instance, allow to screen cells or compounds, enable cell recruitment and delivery, control mechanical or environmental properties, structure tissue or provide functionalized surfaces, or enable gene delivery [233]. Therefore, regenerative medicine is linked to engineering, nanotechnology, biomaterial development, and also optics, utilizing techniques such as microfluidics, high-throughput screening or sequencing, bioprinting, structured cell-growth, or 3D (scaffold-based) cell culture (see Figure 7) [233–237].

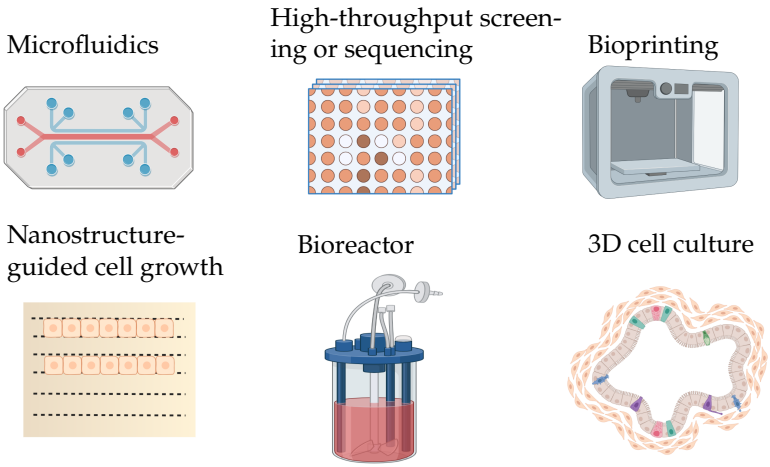


Figure 7: Non-optical enabling technologies in regenerative medicine. Several techniques help to develop and test novel biomaterials, for instance, to enable small volume screening via microfluidics, bioprinting of tissue, or structured cell growth. Created with BioRender.com.

Microfluidics, for instance, is the manipulation of fluids at small scales. It enables miniaturization and parallelization while providing the ability to create compartments and gradients at high resolution [238]. This leads to lab-on-a-chip approaches, which can be used on a single-cell level but even on a large tissue scale to study regeneration and regenerative approaches in defined microenvironments [238, 239]. Due to the parallelizability, microfluidics is also well-suited for high-throughput screening. The development of high-throughput techniques, which allow to identify specific phenotypes of stem cells or to test the influence of material properties on stem cells, is another pillar of enabling techniques [240, 241]. This is also in line with new developments in RNA sequencing, which employ barcoding and allow transcriptome analysis at a single-cell resolved level on large scales [242, 243]. Bioprinting utilizes inkjet technology, microextrusion, photopolymerization, or laser systems to assemble tissues [235]. The assembly can be biomimetic as an attempt to directly replicate a

given environment, or be based on later self-assembly or assembly of micro-tissues. It also involves the creation of new bioinks, which can be based on hydrogels, be fully synthetic, or natural [235]. Structured cell growth is largely inspired by nanotechnology. Nanopatterning or modified nano-surfaces can modulate cell adhesion and interaction, or as 3D nanofibers help to mimic tissue properties [236]. Nanoparticles can also be used as drug delivery agents [236]. Bioreactors can help in large-scale cell expansion or as microfluidic bioreactors in lab-on-a-chip systems [244].

Optical enabling techniques

Optical technologies also offer several enabling technologies for regenerative sciences [245]. Broadly, these can be categorized in techniques, which allow monitoring of the steps involved in the generation of tissue, and its implantation and in methods, which can be applied to modify the cells or tissue of interest (see Figure 8).

Optics can offer several enabling technologies for regenerative sciences. This includes monitoring techniques, for example, label-free imaging, but also techniques to modify tissue.

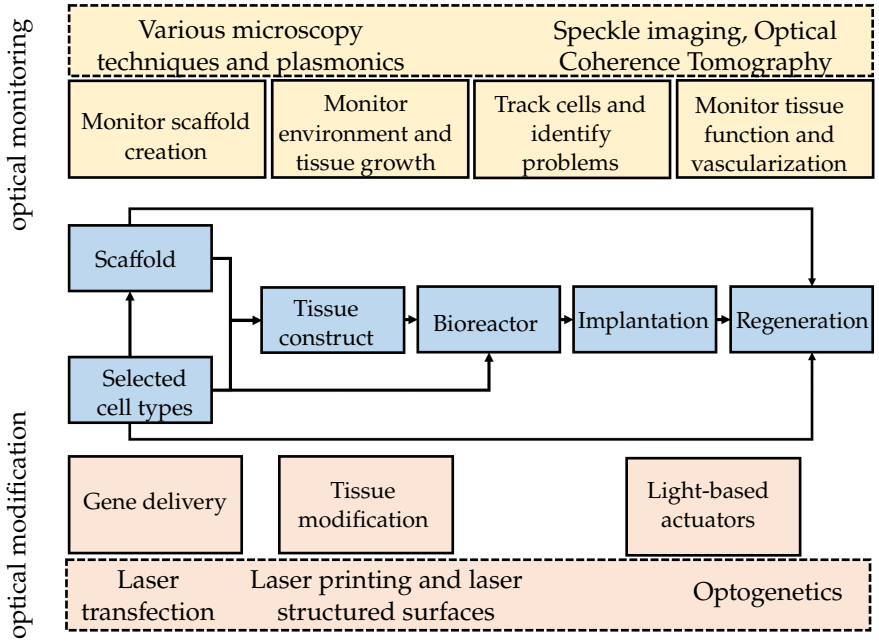


Figure 8: Optics can contribute to the engineering principle in regenerative medicine at various points. Broadly, monitoring can allow visualizing cells, scaffold creation, tissue growth, and tissue function, while modification techniques allow, for example, gene delivery or laser printing. Figure based on [245] (Chapter 1).

Monitoring is important because of the complexity of the individual components in tissue engineering and their interplay. Next to various microscopy techniques, such as multiphoton microscopy, in particular, label-free image approaches are important because they do not require staining of the tissue. These include Fourier-Transform Infrared Spectroscopy, Raman Spectroscopy, and CARS imaging [246]. Analysis of vascularization and function can be fulfilled by laser speckle imaging or optical coherence tomography [247]. Even microscopic rheological properties can be investigated via speckle imaging or Brillouin microscopy [248, 249].

To modify tissue, several optical techniques exist. Optogenetics, as discussed in section 2.5, can yield cells with light-sensitive membrane channels. Such options are discussed for optical pacemakers [250]. To deliver DNA, RNA, or proteins to cells of interest, laser transfection can be applied. Next to femtosecond laser transfection, which can only target a single cell at a time (see section 2.5), high-throughput techniques are under development. This includes approaches, which use nanoparticles to concentrate light and enable membrane perforation [251–254]. Laser-based printing of cells can be utilized to dispose cells on a surface in a well-defined architecture [255]. Two-photon polymerization enables the creation of surface structures with a resolution of several ten nanometers [256].

Novel ideas in regenerative medicine aim to focus on self-organizing cell behavior in stem cell-derived cells. This is motivated by the observation that the addition of growth factors or small molecules might not be fully sufficient to produce adult-like mature cells. During self-organization, cells assemble to a structure with a role that is commensurate with its *in vivo* functional role [257]. Therefore, tissue engineering procedures need to imply the feedback from cells during tissue formation and maturation [257]. However, the use of cells' own internal programs for the spontaneous generation of complex tissues is not mutually exclusive and can be combined with traditional tissue engineering [258]. The realization of this idea requires a much better understanding of the dynamics of cytosystems, in particular, multiscale dynamics of cellular interactions [258] - *which can be realized using optical techniques such as microscopy and optical manipulation*.

3 Outline of this habilitation thesis

This habilitation thesis builds upon the central idea of using light to better understand cellular repair and regeneration. Furthermore, it will address technology developments, which can help to enable regenerative therapies.

In the own preliminary work for this habilitation, a connection between femtosecond laser imaging (multiphoton microscopy) and manipulation of cells has been analyzed. A process, which is called high-order photobleaching and occurs during multiphoton microscopy, was investigated [259]. It was found that this process is similar to femtosecond laser nanosurgery and also involves the formation of a so-called low-density plasma of free electrons. Consequently, the transition between imaging and laser manipulation is smooth and requires careful parameter optimization. This is, in particular, true for the applied staining procedures as fluorophores might also contribute to the formation of seed electrons during multiphoton ionization.

Furthermore, a femtosecond laser system was applied to probe the delivery of molecules into cells (optoporation). In a research stay at St. Andrews university, the optoporation of BY2-tobacco cells was demonstrated [260]. A research stay at Cornell University, during the doctoral thesis, which was focused on *in vivo* application of femtosecond laser manipulation yielded for the first time the demonstration of femtosecond laser optoporation in the mouse brain of a living mouse (unpublished data, see Figure 7).

As an enabling technology for regenerative sciences, gold nanoparticle-mediated laser transfection was established within the doctoral thesis [261]. Compared to femtosecond laser transfection, which only allows targeting of a single cell per time, it could reach high throughput by irradiating gold nanoparticles, which confined the laser irradiation to the cell membrane. Thereby delivery of different molecules including large dextrans or proteins was reached [251, 262]. It was combined with modified surfaces of silica particles or glass surfaces to enhance the delivery [263, 264]. Additionally, first experiments on the underlying mechanisms and an extension of this technique to cell stimulation were performed [265–267].

The preliminary experiments show, that optics can be highly beneficial for enabling novel technologies in regenerative sciences, as detailed in Chapter 2. This can be even extended by combining optics more with material sciences, for example, via the use of a defined cell seeding or application of different materials. Ultimately, this can lead to the development of novel cell modification techniques. Nevertheless, also a fundamental understanding of the associated processes is necessary. Optics can even serve as the tool to enable a general understanding of cell repair and regeneration. However,

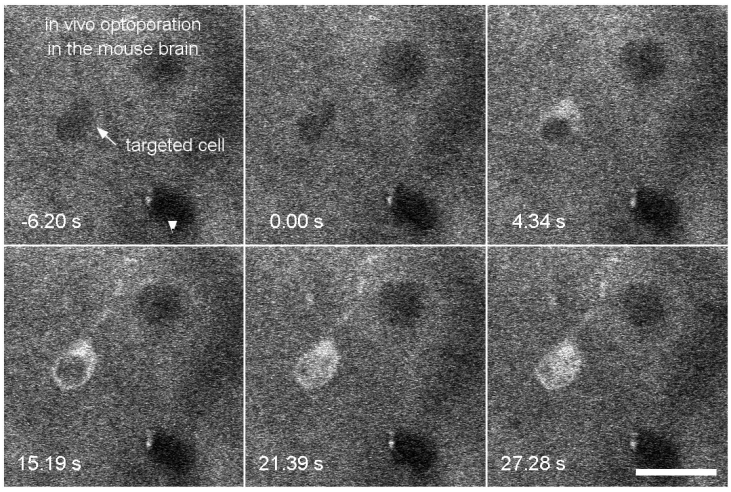


Figure 7: Femtosecond laser-based delivery of dye molecules (FITC dextran) into a single cell in the brain of a living mouse. The brain was loaded with FITC dextran via a micromanipulator and imaged via multiphoton microscopy. A femtosecond laser amplifier system was applied to perforate a single cell. Successful perforation is visible by dye inflow over time. This figure is reproduced from my own doctoral thesis [261] and was acquired in the laboratory of Prof. Schaffer (Cornell University) in collaboration with P. Gadamsetty and S. Hu. Scale bar 20 µm.

both require close observation of the processes and also a highly-confined method for manipulation. This habilitation will describe the establishment, characterization, and application of several optical techniques for cell imaging and manipulation and also show novel applications of optics as enabling technology. In detail, the following aims are addressed in this habilitation:

1. How can cellular imaging be optimized, such that cellular structures can be imaged in the complex environment associated with regeneration? Is it possible to achieve longitudinal *in vivo* imaging of regenerative processes?

2. Can optical manipulation, in particular femtosecond laser nanosurgery, be used to get a comprehensive view of cellular repair processes, with a biophysical and biochemical emphasis? Can it be extended to understand cellular interplay in complex *in vitro* structures like organoids or even *in vivo*?

3. Can previous work on gold nanoparticle-mediated laser manipulation be extended to other modalities to enhance the delivery of molecules into cells for future regenerative therapies?

In chapter 4 the application of light on a single cell level will be addressed. In particular, cellular repair of cardiomyocytes, which are important for regenerative approaches in the heart, is investigated. Also, light-created systems to define cell growth and analyze cell communication are described.

Chapter 5 is focused on visualization and manipulation on a tissue scale. An approach, which uses fiber optics in combination with neural network-based image analysis is explained. This approach can allow longitudinal *in vivo* imaging. Additionally, femtosecond laser manipulation for single-cell removal and long-term imaging of the cellular response is demonstrated in organoids and *in vivo*.

Chapter 6 shows new light-based tools for regenerative medicine. Emphasis is put on gene editing via laser transfection and new materials, which in interaction with optics, can enable the delivery of molecules into cells.

Chapter 7 closes this habilitation with a final conclusion.

How light can help to understand cellular mechanisms

4

This chapter will address the application of light to reach defined cell manipulation. First, femtosecond laser manipulation will be discussed, with an emphasis on the influence on the cell state. It will be applied for studying repair in cardiomyocytes. Additionally, this chapter will present light-created systems that enable to guide cell growth or analyze cell communication.

4.1 How does the cell state influence femtosecond laser nanosurgery?

The basic physics of femtosecond laser nanosurgery are well understood (see Chapter 2). The simultaneous absorption of several photons leads to the ionization of molecules in the focus if the sum of the photon energies is sufficient to overcome the bandgap [193]. This process is called multiphoton ionization and can be well-tuned by using femtosecond laser irradiation. Own preliminary work has shown that it is closely related to high-order photobleaching and a smooth transition between both occurs at a given laser intensity [259]. The ionization process leads to the formation of free electrons that are involved in bond breaking and the formation of reactive oxygen species [193]. Typically, the ionization in biological specimens is modeled for water as a central molecule. However, molecules such as biomolecules or dyes might influence the bandgap [268]. Additionally, from the biological perspective, the current state of the cell might need to be taken into account.

Key Publication 8: Hagenah et al., 'Effects of cell state and staining on femtosecond laser nanosurgery', *Journal of Biophotonics* (2018)

In ***Key Publication 8*** C2C12 myoblasts and myotubes were manipulated using femtosecond laser nanosurgery. This served to elucidate the role of the cell (differentiation) state on femtosecond laser manipulation. C2C12 myoblast cells are a skeletal muscle cell line that undergoes myogenic differentiation upon lowering serum concentration to form a multinucleated syncytium of myotubes. Cell nanosurgery with different cutting lengths was applied randomly in the cytosol in ***Key Publication 8***, with the hypothesis that differentiated myotubes might be more sensitive to the damage. This was confirmed as remodeling of the cytosolic areas was longer and failed in more cases leading to cell death. Shelby et al. analyzed the survival of Chinese hamster ovary cells, neuroblastoma cells fused with glioma cells, and murine embryonic stem cells after cell manipulation using a 3 ns pulsed nitrogen laser at 337 nm. They used the laser to remove either mitochondria or lysosomes from the cells and found very different survival rates [270]. This confirms a strong dependence of laser-based cell manipulation on the cell type and state.

Additionally, based on Kuetemyer et al. it can be speculated that staining would also influence nanosurgery [268]. Consequently, ***Key Publication 8*** also evaluated differences between unstained and stained cells. Unstained cells exhibited NADH (nicotinamide adenine dinucleotide signal) autofluorescence using multiphoton microscopy. Staining was performed using MitoTracker Red to allow similar signals. The survival rate of stained and unstained myotubes and myoblasts was similar, while the remodeling was longer in stained cells. This indicates that nanosurgery could be influenced by the staining, probably because dye molecules can contribute to additional seed electrons [268]. To derive the contribution of dye molecules, one has to account for the ratio of water and dye molecules. Assuming a volume of 1 μm^3=1 fl, this corresponds to $3.3 \cdot 10^{10}$ water molecules. Typical dye loading results in about $1 \cdot 10^8$ dye molecules per cell, which approximately equals $2.5 \cdot 10^4$ dye molecules per femtoliter [137]. Therefore, the contribution of dye molecules to multiphoton ionization might be small. However, as processes such as high-order photobleaching are probably also connected to low-density plasma formation [259], their role is not negligible.

4.2 Understanding cellular repair - novel insights in (cardio-) myocytes

If correctly established, femtosecond laser nanosurgery can help to better understand cellular repair. Based on the above-mentioned parameter optimization, this habilitation focused on the sarcomeric cytoskeleton in cardiomyocytes. As sarcomeres are the smallest contractile unit of each myocyte, such findings can help to better understand muscle physiology and pave the way to novel therapies. The lateral borders of each sarcomere are called Z-disc ("Zwischenscheibe"). The Z-disc is a nodal point for all signaling in a cardiomyocyte and is involved in mechanosensation and mechanotransduction [271]. The Z-disc main component is α-actinin, but it consists of hundreds of different proteins. Due to its broad function, its integrity is mandatory for the sarcomere function [271–273]. Several sarcomeric disorders are based on Z-disc irregularities and can result in severe cardiomyopathies [271, 274, 275].

To investigate Z-disc function, knock-out or knock-down models, patient-specific cells, or chemical/pharmacological treatment can be applied. These usually affect the whole cell and do not allow an isolated view on a single Z-disc inside the complex sarcomeric cytoskeleton of a cardiomyocyte. In ***Key Publication 7*** femtosecond laser nanosurgery of single Z-discs was developed (see Figure 8). It was applied to remove a single Z-disc in rat neonatal cardiomyocytes and in human pluripotent stem cell-derived cardiomyocytes (hPSC-CMs). The Z-discs were labeled by fluorescent α-actinin. The removal was confirmed by antibody staining and comparison to photobleaching. After careful deviation of appropriate laser parameters, it was determined that the viability of cardiomyocytes is not significantly

Key Publication 7: Müller et al., 'Femtosecond laser-based nanosurgery reveals the endogenous regeneration of single Z-discs including physiological consequences for cardiomyocytes', *Scientific Reports* (2019)

affected by the loss of a single Z-disc. Dependent on the cell type, the time to restore a regular Z-disc pattern was 5 h for hPSC-CMs or 24 h for neonatal cardiomyocytes. Calcium homeostasis was not changed after Z-disc loss.

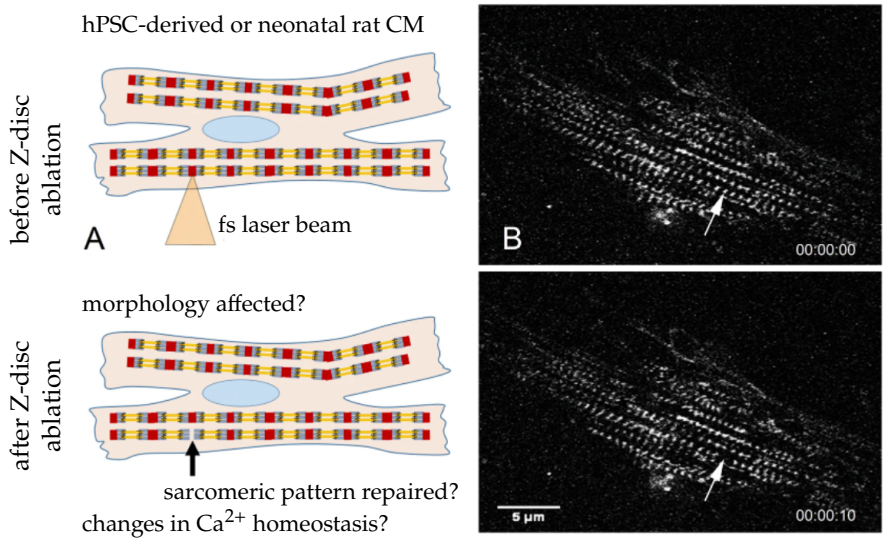


Figure 8: Applications of femtosecond laser nanosurgery for Z-disc ablation in cardiomyocytes to understand cadiomyocyte repair. In (A) the process is illustrated, while (B) provides multiphoton microscopy images of a fluorescent α-actinin expressing neonatal rat cardiomyocyte, which was manipulated. The image was taken and modified from Key Publication 7, Müller et al., 'Femtosecond laser-based nanosurgery reveals the endogenous regeneration of single Z-discs including physiological consequences for cardiomyocytes', Scientific reports (2019) under a CC BY 4.0 license. The font of the labels was adapted.

In ***Key Publication 4*** the evaluation of Z-disc loss after femtosecond laser nanosurgery was extended to multiple Z-discs. Next to single Z-disc ablation, three, five, or ten Z-discs were removed. These were either distributed randomly around the cell or a connected pattern was chosen for ablation. Ablation of ten or five Z-discs led to cell viabilities below 20% or below 51%, respectively. Loss of three Z-discs yielded viabilities above 65%, for both randomly ablated Z-discs and Z-discs ablated in a connected pattern. The number of Z-discs per cell and cell area had no influence on the outcome. Additionally, the damage location was not related to cell survival. Laser ablation of a Z-disc led to a sarcomere shortening of 6 to 7% in relation to the initial sarcomere length. Additionally, 2 h post-ablation, the myofibrillar alignment was significantly altered.

Key Publication 4: Müller et al., 'Evaluation of laser induced sarcomere micro-damage: Role of damage extent and location in cardiomyocytes', *PloS one* (2021)

In ***Key Publication 2*** Z-disc damage was evaluated on a transcriptional level in hPSC-CMs. Upon damage, expression of Filamin-C (FLNc), α-actinin 2 (ACTN2), and nuclear factor-κB (nf-κB) was upregulated. Further cardiac stress genes or sarcomeric cytoskeletal genes showed no changes of expression. Additionally, the expression of the contraction-related genes cardiac muscle troponin T (TNNT2) and troponin I (TNNI3) was downregulated. While the changes in nf-κB are probably cell stress-related, ACTN2 upregulation is likely related to Z-disc repair. FLNc is also involved in sarcomere repair and recruited to damage sites, which explains its upregulation [279, 280]. The changes in TNNT2 and TNNI3 might affect

Key Publication 2: Müller et al., 'How Localized Z-Disc Damage Affects Force Generation and Gene Expression in Cardiomyocytes', *Bioengineering* (2021)

force generation. Therefore, in ***Key Publication 2*** traction force microscopy was used to determine changes in cellular traction forces upon Z-disc removal. The traction forces were determined in the relaxed and contracted state of each cardiomyocyte. A significant change of maximal and minimal traction force was observed upon femtosecond laser-based Z-disc removal. This is likely due to the high interconnectivity of each Z-disc within the cardiomyocyte, which leads to a decrease in force generation.

The results on cell repair further show the good applicability of femtosecond laser manipulation for precise cell manipulation on the subcellular level. Several other applications of subcellular laser surgery using a variety of laser systems are presented [281–283], but other laser systems do not reach the precision of femtosecond laser manipulation. Similar studies, going into detail on the biological outcome of femtosecond laser manipulation on the subcellular organelle level are still limited. Forer et al. recently demonstrated its application to study and disconnect the mechanical connection between separating chromosomes [284]. A similar impressive publication addressed the Golgi complex and its biogenesis but using sub-nanosecond UV-laser pulses [285]. Additionally, microtubules and actin fibers have been analyzed in detail [286–288].

Several improvements might benefit the applicability of femtosecond laser nanosurgery. If it is used for optoporation, plasmonic nanoparticles might enhance the process [254, 289]. Additionally, it can be combined with microfluidics in "on-a-chip" approaches, for instance, to study nerve regeneration [290]. Ronchi et al. propose to combine cell nanosurgery with cell patterning, as already accomplished in Tangemo et al. [282, 285]. This can lead to a standardization of cell shape and intracellular organization and thus enhance reproducibility, yield to equal microenvironments, or serve to concentrate or displace organelles [282].

4.3 Light created systems to define cell growth and analyze cell communication

The application of light can help to structure cell growth, as detailed in Chapter 2. The use of femtosecond lasers bears in this case similar advantages as those in laser manipulation or imaging of cells. It has a high penetration depth and high intensities that can be applied to evoke nonlinear processes. Furthermore, it has the advantage of non-contact fabrication, which can decrease the risks of contamination.

In ***Publication 15*** micro through-holes were fabricated through laser ablation in poly-L-lactic acid and C2C12-Chr2 cells were seeded on this structure. The cells showed enhanced adhesion and alignment along with the structures. Additionally, the myoblast fusion was enhanced, which indicates an influence on differentiation to myotubes. As poly-L-lactic acid is a biodegradable polymer, such a light-created scaffold might benefit regenerative approaches as a special scaffold construct. Furthermore, it

Publication 15: Takayama et al., 'Myoblast adhesion and proliferation on biodegradable polymer films with femtosecond laser-fabricated micro through-holes', *Journal of Biophotonics* (2020)

might be combined with femtosecond laser nanosurgery as it can help to enhance standardization. Compared to other work, ***Publication 15*** used a direct modification of the hydrogel. Other possibilities include immobilizing cell adhesive ligands in hydrogels using two-photon laser scanning photolithography [292] or to use absorbing nanoparticles to allow hydrogel modification [293]. Recently, Xiong et al. also used a direct femtosecond laser-based writing method in a cell-laden hydrogel and observed two possible regimes, gel densification, and gel ablation [294]. The cell viability remained at 90%, despite direct writing in the gel [294].

In ***Key Publication 3*** another light-based system for cell manipulation was defined. It enabled to probe cell communication between spatially separated cell populations. It was based on the expression of a light-sensitive ion channel, the channelrhodopsin variant CheRiff, in mouse cortex or hippocampal neurons. These neuron populations were spatially separated over a distance of 1 mm and allowed to connect via dendrites over several days. Optogenetic blue-light activation of CheRiff triggered a signal, which propagated from the excited neuron population, either cortex or hippocampal neurons (actuator), to the spiral-ganglion neurons (receiver). This was visualized via calcium imaging. As both, actuator and receiver are completely based on light interaction, they are non-invasive. A similar strategy was applied by Jia et al. in cardiac optogenetics. They used non-excitable human embryonic kidney (HEK) cells, which expressed the light-sensitive ion channel Channelrhodopsin-2 (HEK-ChR2 cells). Gap junctional coupling of these cells as "tandem" partners to cardiomyocytes allowed indirect cardiomyocyte excitation via optogenetics [296]. This could be even extended to the development of multi-strip cardiac muscle by co-cultivation of cardiomyocytes, fibroblasts, and HEK-ChR2 cells [297]. Such an approach might be beneficial in tissue engineering, as cardiomyocytes are difficult to transfect or transduce, while cell lines such as HEK cells can be well genetically modified [297].

Key Publication 3: Heeger et al., 'Probing interneuronal cell communication via optogenetic stimulation', *Translational Biophotonics* (2021)

In another application of cell manipulation, light can be combined with nanoparticles that absorb or scatter and confine the light. Such an approach was already applied in own preliminary work in HL-1 cells, a cardiomyocyte-like cell line, to trigger cell stimulation [267]. Laser irradiation of membrane-attached gold nanoparticles, "plasmonic laser stimulation", led to a calcium rise in the directly irradiated cell and a calcium wave propagated along the surrounding cells. In ***Publication 20*** this approach was extended to neuronal cells, with emphasis on the role of calcium in the irradiated cell. It demonstrated via calcium imaging and analysis of lipid peroxidation, that upon irradiation initial calcium is released from the endoplasmic reticulum and flows in through the wounded membrane. The inflow can potentiate the intracellular calcium release. Additionally, mitochondria release calcium. Dependent on the cell environment this can potentially have fatal effects on cell viability. This shows, that light-created systems need tight control of all parameters, in particular in interplay with further factors, such as nanoparticles.

Publication 20: Johannsmeier et al., 'Gold nanoparticle-mediated laser stimulation induces a complex stress response in neuronal cells', *Scientific Reports* (2018)

4.4 Conclusion and outlook

The last chapter has shown the good applicability of light, in particular, ultrashort pulsed laser systems for a defined manipulation on a cellular level. Femtosecond laser nanosurgery is well suited to understand cellular repair processes and this chapter has shed light on cardiomyocyte repair. While the precision of femtosecond laser nanosurgery is already very high, ideas to increase it even further in the future are existent. A first study by Pospiech et al. has demonstrated super-resolved femtosecond laser nanosurgery [299]. It used phase filtering with a programmable phase modulator to increase the resolution about 30%. Gold nanoparticle based cell surgery using femtosecond laser pulses might also be considered as "super-resolved" as the gold nanoparticles confine the light via field-enhancement in their vicinity ("nanolens-effect") [289]. Further studies of super-resolved nanosurgery are yet missing.

Resolution is determined by the wavelength and an interesting idea would be to realize visualization and manipulation at very short wavelengths, for instance, in the X-ray regime. X-ray microscopes have already found a way to biological applications, for example, at synchrotron facilities [300, 301]. The applicability on a cellular level with fixed cells is challenged by radiation damage that is connected to changes of the cellular biochemical profile via bond-breaking [302]. In live cells, a dose of 100,000,000 Gray would be necessary to obtain a nanometer resolution, which is magnitudes above any survivable value [303]. However, new laser systems are paving the way to realize live-cell X-ray microscopy. Free-electron lasers can be continuously tuned to wavelengths from X-ray to terahertz irradiation and enable laser parameters that cannot be realized by conventional lasers [304, 305]. This has enabled the development of femtosecond pulse X-ray sources and their application for diffractive imaging [302, 306, 307]. In an approach called "diffraction before destruction," the samples are imaged in their native state because the use of femtosecond pulses enables to overcome the speed of damage formation, such that samples only get destroyed after imaging [303, 307, 308]. This has recently even been applied in live cells, either aerosolized or embedded in a micro-liquid enclosure [303, 308]. As also novel labels, for example, genetically encoded X-ray probes are under development [309], it can be well assumed that X-ray microscopy in cell biology comes into reach. It can be speculated that this also opens novel possibilities for ultraprecise manipulation, especially to intervene with fast processes, which might be observed before sample destruction. A study by Beyerlein et al. recently demonstrated that X-ray exposure of water can cause a phase change from liquid to plasma state, which is associated with the destruction of the biological structure [310]. It can be speculated that such regimes might be suitable for manipulation in the future.

In addition to aspects of femtosecond laser nanosurgery, light-based systems using defined cell seeding, optogenetics, or plasmonic cell stimulation

were presented. The future of this field is wide and individual developments in nanotechnology, physics, and biology might contribute to its progress. A very interesting future application is to use light to control biological systems beyond the scope of optogenetics. Light-based circuits in synthetic biology can address potential needs in regenerative medicine by utilizing ideas from cellular interplay. A pioneering example by Ye et al. used light-activated melanopsin to trigger a synthetic signaling pathway in HEK293T cells via the nuclear factor of activated T cells to control the expression of alkaline phosphatase or glucagon-like peptide 1. In the latter case, this allowed to control insulin levels in type II diabetic mice [311]. Folcher et al. created a mind-controlled gene switch in which a near-infrared LED was remotely controlled via brain wave signals from an electroencephalography brain-computer interface. Near-infrared light activated a synthetic signaling pathway via bacterial diguanylate cyclase, which led to the expression of secreted alkaline phosphatase [312].

Further light-based circuits are currently evolving [313, 314]. A major limitation of such applications is the low penetration depth of visible light into tissue. The demonstration by Folcher et al. shows a good example of applying near-infrared light-sensitive switches, which provides a much better tissue penetration [312]. However, also other switches, such as the above-mentioned melanopsin can be applied in the future as upconverting nanoparticles or multi-photon optogenetics enable novel combinations of near-infrared light with visible-light-activated channels [315, 316].

Applying light on the tissue scale - understanding repair and regeneration

5

Imaging plays a key role to understand biology on a cellular scale. Several microscopic imaging modalities are available to allow the visualization of native tissue in large three-dimensional volumes. This chapter will first outline some applications of imaging to study or enhance regenerative processes. Second, imaging strategies for longitudinal imaging in a mouse model are presented. Furthermore, this chapter extends the application of laser-based cell surgery to a tissue scale, both *in vitro* in organoids and *in vivo* in the mouse.

5.1 Imaging to understand cell alignment in tissue

The application of tissue imaging modalities, such as confocal imaging or multiphoton microscopy is of key interest to understand cell dynamics, in particular in tissue engineering. In ***Publication 12*** multiphoton microscopy was applied to better understand the formation of matrix-free human pluripotent stem cell aggregates. In particular, it allowed investigating the process of cavity formation dependent on external conditions.

In ***Publication 13***, ***Publication 14***, and ***Publication 19*** confocal or multiphoton imaging were used to understand cell distribution and alignment in endothelial networks. Different scaffold materials, macroporous polydioxanone, collagen I, or collagen-agarose served as the basis for cell seeding. Multiphoton imaging in ***Publication 13 and 18*** allowed to determine important network parameters such as branching and nodes. The advantage of multiphoton microscopy in such settings is its high penetration depth, such that three-dimensional reconstructions of networks are possible. In ***Publication 17*** multiphoton microscopy served to evaluate the biodistribution of magnetic nanoporous silica nanoparticles in fresh tissue sections. Such nanoparticles can enable drug delivery in implants or regenerative medicine and understanding their interplay with cells via imaging is consequently of high importance.

Publication 12: Manstein et al., 'High density bioprocessing of human pluripotent stem cells by metabolic control and in silico modeling.', *Stem Cells Translational Medicine* (2021)

Publication 13: Heene et al., 'Vascular network formation on macroporous polydioxanone scaffolds', *Tissue Engineering Part A* (2021)

Publication 14: Ichanti et al., 'Characterization of Tissue Engineered Endothelial Cell Networks in Composite Collagen-Agarose Hydrogels', *Gels* (2020)

Publication 19: Andrée et al., 'Formation of three-dimensional tubular endothelial cell networks under defined serum-free cell culture conditions in human collagen hydrogels', *Scientific Reports* (2019)

Publication 17: Janßen et al., 'Biodistribution, biocompatibility and targeted accumulation of magnetic nanoporous silica nanoparticles as drug carrier in orthopedics', *Journal of Nanobiotechnology* (2020)

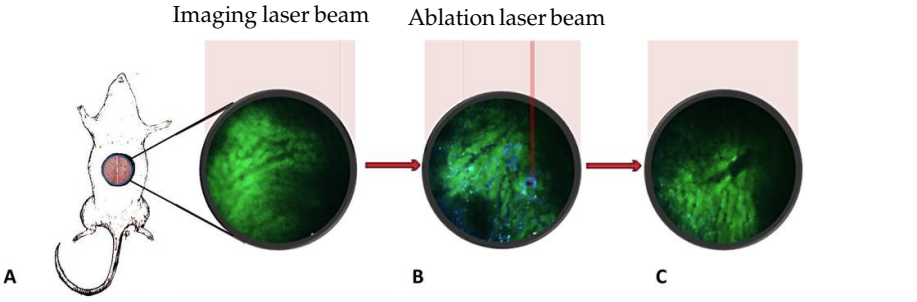
5.2 Accessing tissue *in vivo* using imaging windows and digitally augmented fiber-based microscopy

As detailed in Chapter 2, *in vivo* based imaging in rodent models is limited by the accessibility of the tissue of interest if cellular resolution is desired. In accordance with the guiding principles of animal welfare in experiments, the three Rs (reduce, replace, refine), it is also mandatory to refine *in vivo* imaging experiments such that a low number of animals

is necessary. Imaging the same mouse in repeated imaging sessions over a defined time course will reduce the number of animals and might also enhance data quality. Consequently, imaging modalities that require sacrificing the animal after imaging are not well-suitable. Additionally, such longitudinal imaging experiments would allow gaining better data on events and processes as the experimental heterogeneity is reduced. For clinical translation of the obtained results, this is of high importance.

One possibility for longitudinal *in vivo* imaging is the use of imaging windows. In ***Key Publication 6*** longitudinal *in vivo* imaging of the mouse liver was evaluated based on an abdominal imaging window (see Figure 9). This technique was previously demonstrated in the intestine, liver, and pancreas by Ritsma et al. [133–135]. Hepatocytes could be well visualized via Rhodamine 6G injection and Fluorescein Isothiocyanate (FITC)-dextran was used for labeling of vessels. Longitudinal imaging on several days of the same region could be realized. The animals tolerated the procedure well, which was based on the mouse grimace scale that was evaluated during routine mouse inspection. In particular, imaging was also combined with manipulation as detailed in 5.3.

Key Publication 6: DeTemple et al., 'Longitudinal imaging and femtosecond laser manipulation of the liver: How to generate and trace single-cell-resolved micro-damage in vivo.', *PloS one* (2020)



Mouse with implanted AIW and Rhodamine 6G staining of the liver before single cell ablation

Change in liver tissue directly after manipulation

Full regeneration of the tissue region after 24 h

Figure 9: Applications of longitudinal imaging and femtosecond laser nanosurgery *in vivo* in a mouse with implanted liver abdominal imaging window (A). (B) Multiphoton microscopy image after application of a targeted femtosecond laser pulse (green: Rhodamine 6G, blue: FITC-Dextran) and follow-up analysis of the manipulated region (C). The image was taken from Key Publication 6, DeTemple et al., 'Longitudinal imaging and femtosecond laser manipulation of the liver: How to generate and trace single-cell-resolved micro-damage in vivo', PloS (2020) under a CC BY 4.0 license. The font of the labels was adapted.

A limitation of imaging windows is the necessity to surgically fix the tissue of interest to the window, for instance, via cyanoacrylate glue. In particular, this limits the applicability of imaging windows for deep organ cellular imaging. In ***Key Publication 5*** another imaging route was used. An imaging fiber bundle with 30.000 fibers coupled to a custom-built fluorescence microscope was applied for abdominal imaging in mice in single final imaging sessions (see Figure 10). Such fiber bundle microscopes have been investigated previously and been demonstrated in combination with wide-field or laser scanning (confocal) fluorescence microscopy as detailed in Chapter 2. A challenge in establishing such technologies is to address the cost for future users. A confocal laser-scanning setup would enable better image quality but has high cost as a laser system and sophisticated

Key Publication 5: Bahlmann et al., 'Establishment of a guided, in vivo, multi-channel, abdominal, tissue imaging approach', *Scientific Reports* (2020)

scanning optics are necessary. In ***Key Publication 5*** the microscope setup consisted of an objective, a LED light source, four-band dichroic, excitation, and emission filters, and a sensitive CCD camera. This enabled to reduce the setup cost to below 30.000 euros.

To obtain high image quality, a convolutional neural network based on the U-net architecture was trained. It was based on the CSBDeep toolbox for content-aware image restoration [157, 158]. Confocal microscopy images of the same tissue served as training data. Three different modalities for the incorporation of the fiber-bundle architecture into the image restoration were compared. The first used blurred fiber-bundle images for restoration, the second added a synthetic fiber-core structure to the training data, and the third overlapped the original bundle structure with the training data. The combination of the original bundle structure with training data yielded the best result and for some organs, confocal microscopy-like images were obtained after reconstruction of the fiber bundle images.

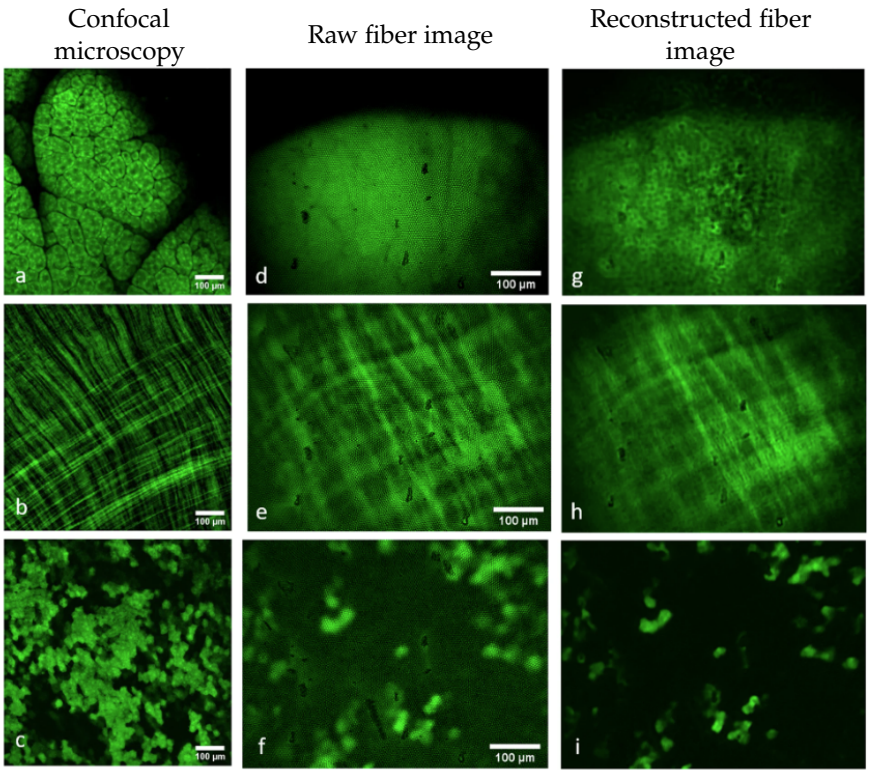


Figure 10: Confocal and fiber microscopy images from different ex-vivo imaged GFP mouse organs. The images (a–c) were acquired using a confocal microscope, (d-f) using the fiber imaging setup, and (g-i) were reconstructed using neuronal networks. The organs pancreas (a,d,g), jejunum (b,e,h) and liver (c,f,i) are shown. The image was taken from Key Publication 5, Bahlmann et al., 'Establishment of a guided, in vivo, multi-channel, abdominal, tissue imaging approach', Scientific reports (2020) under a CC BY 4.0 license. The font of the labels was adapted.

5.3 Manipulating cells within their complex environment

In the last chapter, manipulation was presented on a single-cell level. The same principles can be applied in tissue, which gives the option to investigate multicellular dynamics in a native environment. A powerful technique to understand three-dimensional cellular dynamics *in vitro* is organoid culture. As detailed in Chapter 2, organoid culture mimics the complex architecture of *in vivo* organ models. To verify the architecture, it is necessary to identify the cellular composition if organoid protocols are established. Confocal microscopy of murine colon organoids (colonoids) was applied in ***Publication 16*** to help to characterize the cellular composition.

Publication 16: Brooks et al., 'CD14 and ALPK1 Affect Expression of Tight Junction Components and Proinflammatory Mediators upon Bacterial Stimulation in a Colonic 3D Organoid Model', *Stem Cells International* (2020)

The homeostasis of the colon is maintained via constant cell renewal of the epithelial barrier from the stem cell niche. Failure of this principle can lead to colon disease. As each colonic crypt only contains about five to ten stem cells in humans [325, 326] understanding colon cell dynamics on a single cell level is crucial. In particular, failure to repair or maintain the epithelial barrier after cellular damage needs to be well understood. Single-cell damage cannot be realized via knock-out models, chemical treatment, or ionizing radiation. In ***Key Publication 1*** femtosecond laser nanosurgery was established in colonoids. Single-cell damage via nanosurgery was applied in the differentiated cell region, in the proliferative cell region, and in the stem-cell containing crypt (see Figure 11).

Key Publication 1: Donath et al., 'Investigation of Colonic Regeneration via Precise Damage Application Using Femtosecond Laser-Based Nanosurgery', *Cells* (2022)

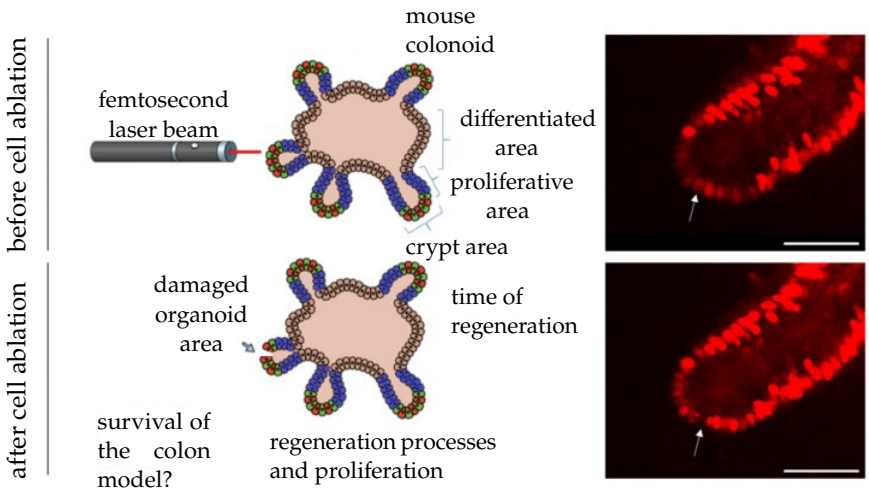


Figure 11: Femtosecond laser nanosurgery in a colonoid. The colonoid is divided into three areas, the crypt zone, the proliferative zone, and the differentiated zone and single cells are removed from these areas. The microscopy images show crypt zone cell ablation in a mCherry-H2a positive colonoid. Scale bar 50 µm. The image was taken and modified from Key Publication 1, Donath et al., 'Investigation of Colonic Regeneration via Precise Damage Application Using Femtosecond Laser-Based Nanosurgery', Cells (2022) under a CC BY 4.0 license. The font of the labels was adapted.

Organoids invaginated the damaged cells into the lumen and regained structural integrity within 24 h after damage. While damage in the differentiated and proliferative region led to no local proliferation changes,

single-cell crypt damage either strongly increased or decreased proliferation in the crypt. Furthermore, local activation of Wnt was observed via a fluorescent biosensor after crypt single-cell damage and gene expression of Ki67 and Survivin (Birc5) was elevated, while expression of Sox-9 was decreased compared to control groups. This points to a large role of Wnt activation in colonoid regeneration after single-cell damage. Similar fast repair processes were also observed in the intestinal niche after laser-based cell manipulation in the mouse *in vivo* by Choi et al. [328].

As demonstrated by Choi et al., single-cell models can even be applied *in vivo* [328]. In ***Key Publication 6*** *in vivo* single-cell manipulation of the mouse liver was achieved via an amplifier femtosecond laser system. In particular, laser parameters to achieve single-cell manipulation were determined and the immediate reaction could be followed. Additionally, it was possible to image the manipulated region via multiphoton microscopy at several days after cell ablation. In the future, such a model can in combination with close observation via imaging, for example, provide insights into the role of the plasticity of differentiated cells in liver regeneration. Depending on the severity of the injury, hepatocyte regeneration in the liver might involve hepatocyte self-duplication, Axin2+ hepatocytes in the pericentral regions, hepatocyte populations with high-telomerase expression, progenitor-like cells, or biliary epithelial cells, and accompanying changes of cell fate via trans-/de-/ or differentiation [329]. As a femtosecond laser allows very specific cell manipulation, single to multiple cells can be manipulated to better understand these processes in a liver lobule.

5.4 Conclusion and outlook

This chapter presented imaging strategies and defined manipulation in complex tissue geometries and *in vivo*. Strategies for potential long-term observations were addressed by developing a fiber-based imaging system and manipulation of the liver through an abdominal imaging window.

In the future, it will be of key interest to better relate imaging data to omics data, in particular transcriptome data. Recently, several groups have demonstrated spatial transcriptomics approaches [330–332]. This can, for instance, be realized via multiple rounds of fluorescent RNA hybridization that allows imaging of up to 10,000 genes in single cells [330]. Another option is to apply spatial barcoding, for example, via a transfer of RNA from tissue to a surface covered with DNA-barcoded beads [331]. Additionally, the development of RNA timestamping, which can give information about multiple past transcriptional events, allows capturing cellular dynamics [333]. This brings spatial-temporal transcriptomics within reach.

To advance longitudinal imaging of regeneration or repair it is desirable to develop techniques with the lowest invasiveness possible. This minimizes the burden to the specimen and also avoids potential influence on the

studied regeneration or repair pathways. Therefore, small imaging probes, such as very thin fibers are desirable. Additionally, three-dimensional visualization of the tissue of interest would be of great interest. Orth et al. have demonstrated that fiber bundles allow gaining depth information. They reconstructed the light-field of the fiber bundle and showed digital refocusing and stereo visualization from a single exposure [334]. As a single exposure is sufficient for 3D visualization, this can greatly enhance image recording speed. Recent advances in light-field microscopy, which employ a view-channel-depth (VCD) neural network for light-field reconstruction, can even enable real-time volumetric visualization [335]. Recently, Stellinga et al. used a 50 µm multimode optical fiber for real-time 3D imaging [336]. The seminal approach combined wavefront shaping and a pulsed laser source to raster scan the field of view at a rate of up to 23,000 points per second. Based on the acquired time-of-flight for each pulse, depth information was calculated. Consequently, in the future smaller endoscopes might become available for imaging.

In vivo, targeted tissue ablation through an imaging window has been already shown in the brain and recently in the intestine [199, 328, 337]. Such manipulation enables to influence cell fate in great detail. In the future, it might be of great interest to analyze cell surgery in the second and third near infrared window. Crotti et al. already examined corneal surgery using a wavelength close to 1650 nm compared to 1030 nm [338]. At the longer wavelength they observed a higher penetration depth, while a good incision quality was still possible. This is supported by simulations by Linz et al. [194]. These indicate that the wavelengths 1300 nm and 1700 nm could be well suitable for cell surgery. At these wavelengths, the optical breakdown threshold at a depth of 200 µm is similar to the one at 800 nm, while at depths of 500 µm and 1000 µm it is even lower [194]. It can be envisioned that *in vivo* cell ablation might be even realized using fiber-based approaches. For instance, Abbasi et al. could demonstrate delivery of laser pulses through an optical imaging fiber bundle [339].

To reduce animal experiments, organoid-based studies such as demonstrated in this chapter might in the future provide a better alternative for functional studies. This requires an advancement in functional validation and reproducibility in organoid systems, which can be reached via engineering principles [340]. To manipulate organoids and study their repair and regeneration, this thesis presented a method to target single cells. Compared to other organoid injury models, for instance, using cryoinjury in human cardiac organoids [171], femtosecond laser manipulation is more precise and allows targeting cells without specific requirements for sterility. However, in the future, it is of interested to select cells based on their characteristics instead of their spatial localization. In particular stem cells are an interesting target concerning regeneration. In the colon, stem cell labeling in organoids can be realized via a recently available ASCL2 reporter [341]. Alternatively, knock-in approaches such as CRISPR-HOT in organoids might help to label cells [342].

Paving the way for developing new light-based tools for regenerative sciences

6

In the second chapter, the contribution of enabling technologies, with a particular emphasis on light-based approaches, was already highlighted. In own preliminary work, in the frame of the doctoral thesis, a technique called gold nanoparticle-mediated laser transfection of cells was established [251–253, 262–266]. This technique used pulsed laser, which lead to the generation of vapor bubbles at membrane-adhered gold nanoparticles. This enables membrane permeabilization. In the frame of this research, delivery of dye molecules [252, 253], siRNA [253], Morpholinos [262], and proteins [251] was demonstrated. Additionally, material combinations, which combined gold nanoparticles and other materials, such as silica particles [264] for enhanced delivery were investigated.

This chapter presents an extension of laser-based delivery techniques. Firstly, delivery in genome editing using gold nanoparticle-based laser transfection is demonstrated, which is a huge step towards wider applicability, in particular, in the context of regenerative sciences. Secondly, the focus is set on the application of novel materials for laser-based transfection.

6.1 Genome editing using laser transfection

Genome editing can enable the direct therapeutic correction of genetic mutations in affected tissues using programmable nuclease platforms such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, or the CRISPR/Cas9 system [343, 344]. The latter technique has since its initial use in genome editing in 2012 evolved in several subsequent publications, found its way in a preliminary study of applying CRISPR-edited cells in patients, and is still constantly enhanced, for example, by minimizing byproducts [345–350].

The efficient and safe delivery of guide RNAs Cas9 and its guide RNAs (crRNA:tracrRNA hybrids) to cells is critical for the application of CRISPR/-Cas9 and novel delivery systems might benefit this direction [164, 351]. In ***Key Publication 9*** the application of gold nanoparticle-mediated laser transfection for CRISPR/Cas9 genome editing was evaluated. The delivery of crRNA:tracrRNA, which targets mouse CCR7, in SpCas9 (Streptococcus pyogenes Cas9) expressing cells was successfully accomplished. Additionally, ribonucleoprotein particles, consisting of crRNA:tracrRNA and SpCas9 were delivered into SC1 cells resulting in knockout efficiencies of 65% in the laser transfected cells. In mouse CD8+ T cells CXCR3 was targeted via delivery of ribonucleoprotein particles which resulted in efficiencies of about 4% in all cells and 25% in laser transfected cells. This well proves the general applicability of gold nanoparticle-mediated laser transfection for

Key Publication 9: Bošnjak et al., 'CRISPR/Cas9 Genome Editing Using Gold-Nanoparticle-Mediated Laserporation', *Advanced Biosystems* (2018)

delivery in gene editing approaches. Recently, Raes et al. demonstrated vapor nanobubble-mediated photoporation for delivery of Cas9 ribonucleoproteins. This technique is similar but uses higher laser fluences. It reported gene knockout efficiencies knock-out levels of up to 34% in primary human T cells [353]. In immunotherapies, T cells are the therapeutic target and nanotechnology can be an advancement in current delivery strategies, also in the context of CRISPR/Cas9 genome editing [354]. Therefore, the combination used in gold nanoparticle-mediated laser transfection could be of key interest for the development of similar, future strategies.

6.2 New materials for laser-based cell transfection

Next to gold nanoparticles, other materials might also be suitable for high-throughput molecule delivery in combination with laser-based irradiation. One option is plasmonic pyramid arrays made of glass and covered with a 50 nm layer of gold [355, 356]. The physical mechanisms of this technique are similar to gold nanoparticle-mediated laser transfection and it can also reach high efficiencies in molecular delivery [355, 356]. However, the associated changes on the cell level might be different, as the cells adhere to the gold pyramids. In ***Key Publication 10*** C2C12 myoblasts and myotubes were analyzed with plasmonic pyramid-based laser transfection. Each pyramid let to a pore size of approximately 20 nm. It was further determined that the actin stress fibers in the cell realign rapidly after laser irradiation but stabilize within 10 min. Additionally, a 10% decrease in cell area was observed. Delivery of molecules only occurred in cells with close contact to the pyramid array. This limits the applicability of plasmonic pyramid arrays with suspension cells, while nanoparticles are free-floating and can attach to every available cell surface. On the other side, this also bears an advantage as nanoparticles can be taken up by the cells, while pyramid arrays are a stable-surface structure. For the future of laser-based delivery techniques, this could consequently enhance safety.

Key Publication 10: Saklayen* et al., 'Analysis of poration-induced changes in cells from laser-activated plasmonic substrates', *Biomedical Optics Express* (2017)

A disadvantage of plasmonic pyramid arrays is the complexity of their manufacturing process. Other materials might be easier to derive. In ***Publication 11*** polymers were irradiated by an 11 ns pulsed laser at a wavelength of 1064 nm. In detail, commercial polyvinyl tape and black polystyrene Petri dishes were used and the delivery of various dextran sizes in adhered HeLa cells was probed. Up to 20% efficiency for 150 kDa dextrans was realized. Fluorescently labeled siRNA was delivered with an efficiency of 9%. The cell viability after laser treatment was in the range of 60%. In total, polymer surfaces are less efficient compared to gold nanoparticles or plasmonic pyramids, but they are much cheaper and readily available. The used laser parameters are in the range for the formation of an acoustic wave, but in ***Publication 11*** no pressure wave was obtained for white polyvinyl polymer. Therefore, the detailed mechanism of delivery still has to be determined and might involve heating and pressure.

Publication 11: Shen et al., 'Intracellular Cargo Delivery Induced by Irradiating Polymer Substrates with Nanosecond-Pulsed Lasers', *ACS Biomaterials Science and Engineering* (2021)

Next to cellular delivery, other applications of the interaction of gold nanoparticles and laser irradiation can be conceived [359]. In ***Publication 18*** the same laser setup that is employed for gold nanoparticle-mediated laser transfection was used for selective cell killing. This was realized by coupling the gold nanoparticles to the C-terminus of Clostridium perfringens enterotoxin, which specifically targets claudin-expressing tumor cells. In particular, spheroid from Caco-2 and OE-33 cells were targeted and with bare nanoparticles, negligible killing was observed in comparison to conjugated nanoparticles. Such an approach might with further development find use in *in vivo* applications, due to its selectivity. Additionally, necrotic cells might attract immune cells that can boost tumor destruction [361].

Publication 18: Becker et al., 'Parameters for Optoperforation-Induced Killing of Cancer Cells Using Gold Nanoparticles Functionalized With the C-terminal Fragment of Clostridium Perfringens Enterotoxin', *International Journal of Molecular Sciences* (2019)

6.3 Conclusion and outlook

Regenerative sciences can largely benefit from new developments in enabling technologies. This chapter has highlighted recent advances in laser-based drug delivery by demonstrating delivery principles, advanced (nano-) material exploration, and an extension to cell killing. If such technologies can reach *in vivo* applications or even pre-clinical or clinical use, is largely dependent on their efficacy but also on their safety.

The safety of nanomedical products is extensively discussed and standard operating procedures for safety evaluation are currently being established [362, 363]. Shape, size, dose, surface, aggregation, sedimentation, and diffusion dynamics are some points that need to be considered in the evaluation of nanoparticles for regenerative medicine [362, 363]. Yet, concerning both efficacy and safety, several nanomedical products have been approved for clinical trials [364]. Nevertheless, the translation of anticancer nanomedicines, which are based on the enhanced permeability and retention effect in tumors, from preclinical cancer models (mouse xenograft models) to cancer patients is still poor and evidence for a better anticancer treatment compared to free drugs is limited [363]. Therefore, it is likely that nanomedicine has to undergo large changes in the future.

The safety of laser-based enabling technologies can be much better predicted, as light can be fully controlled. Laser-based delivery techniques with or without enhancers such as nanomaterials have not yet reached clinical applications. Currently, the number of cell-targeted *in vivo* laser delivery studies is also very limited [365–369]. A promising recent study by Batabyal et al. demonstrated delivery of a multi-characteristic opsin in the degenerated retina of rd1 mice using femtosecond laser transfection. An electric response could be measured from the retina via patch-clamp after explantation upon visual stimulation [368]. Consequently, more studies are needed to make better predictions about the potential future application of laser-based delivery in regenerative sciences, in particular, as the portfolio of competing delivery strategies is large and evolving [370].

Final conclusion: Illuminating the future of regenerative research

7

This habilitation investigated the applicability of several combinations of optics and biomedicine to advance research on cellular repair and regeneration. Imaging and cell manipulation were presented on a single-cell and tissue scale and optics-based enabling technologies for regenerative medicine were presented.

It addressed the question of imaging in a complex environment and longitudinal, repetitive imaging of regenerative processes. It presented different strategies such as imaging through an abdominal imaging window of the liver as well as fiber-based microscopy enhanced via the application of neuronal networks.

Further it presented several applications of femtosecond laser nanosurgery to better understand cellular repair, first by examining potential influences on the nanosurgery process and second by particularly investigating repair of Z-disc loss in cardiomyocytes. Additionally, even complex environments were investigated and single cells were removed *in vitro* in a colon organoid culture to probe regeneration, as well as in the liver of a living mouse.

Finally, this thesis addressed whether previous work on gold nanoparticle-mediated laser manipulation could be extended to other modalities to enhance the delivery of molecules into cells for application in regenerative sciences. In particular, genome editing in cell culture was demonstrated with good efficiencies. Further, a better understanding of the underlying processes and the application of novel materials was explored.

How can optics help to illuminate future directions in regenerative research? The answer to this question is probably connected to the advancement of the field of biophotonics [371], which also encompassed the directions and techniques presented in this habilitation. Marcu et al. predict among other topics two fundamental future directions: to push the limits and to search for rare signals [371].

In relation to regenerative research, pushing the limits might be explained with the following example: Currently, we know that the Yamanaka factors Oct4 (Pou5f1), Sox2, Klf4, and c-Myc allow reprogramming differentiated cells to induced pluripotent stem cells. We also can visualize such transitions microscopically via various techniques and stainings, even in complex environments such as tissue. However, we currently observe the cells and their function. A challenge for later developments would be to concentrate on the action of the Yamanaka factors themselves: Could we one-day image Oct4 transcription factor binding and activation, maybe in a live cell environment? Consequently, this direction goes beyond current super-resolution techniques down to single-molecule applications.

Searching for rare signals can also be well understood in the context of regenerative research. We have nowadays techniques that allow nearly continuous observation of repair processes. For instance, this habilitation presented organoid-based models, imaging and manipulation through an abdominal imaging window, or a fiber-based strategy, which all allow continuous or longitudinal imaging to a certain degree. However, how can we make sure to observe a given signal, for instance, a cell division in the stem-cell niche? We currently rely on fluorescent labels or label-free techniques. With each of these techniques, we make a pre-selection of our desired observation. Additionally, we plan our experiments with defined time points and temporal resolution. Consequently, we might miss rare signals. The challenge to overcome this problem will be important in the future.

The last point is also connected to an upcoming problem. Abulut et al. demonstrated that GFP impairs actin-myosin interactions and might lead to changes in muscle cell function [372]. Recently, it was demonstrated that a widely used actin probe might disrupt the f-actin organization in myocytes if it is over-expressed [373]. While there are strategies to control expression, for example, via inducible promoters, or to tag endogeneous proteins via CRISPR/Cas9, this points to an important effect: The design of our experiments might largely influence the outcome. Even if we push all limits of detection to the fundamental limit and end up in quantum mechanics, we cannot overcome the observer effect [374]. This is a manifestation of the complementarity principle, which was already derived by Niels Bohr: If one quantity of a pair is measured this excludes to limit the other quantity. However, knowledge about both quantities is necessary to understand the system. For light, this principle manifests itself in the wave-particle duality. This fundamental concept of complementarity was also already extended by Bohr to living systems and might, with all possibilities of the future, be one of the large challenges to implement in (regenerative) biology. We need to understand and accept the coupling of the observer to the observed [375].

"The existence of life itself would have to be regarded in biology, both as regards the possibilities of observation and of definition, as no more subject to analysis than the existence of the quantum of action in atomic physics."

— Niels Bohr —

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A

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