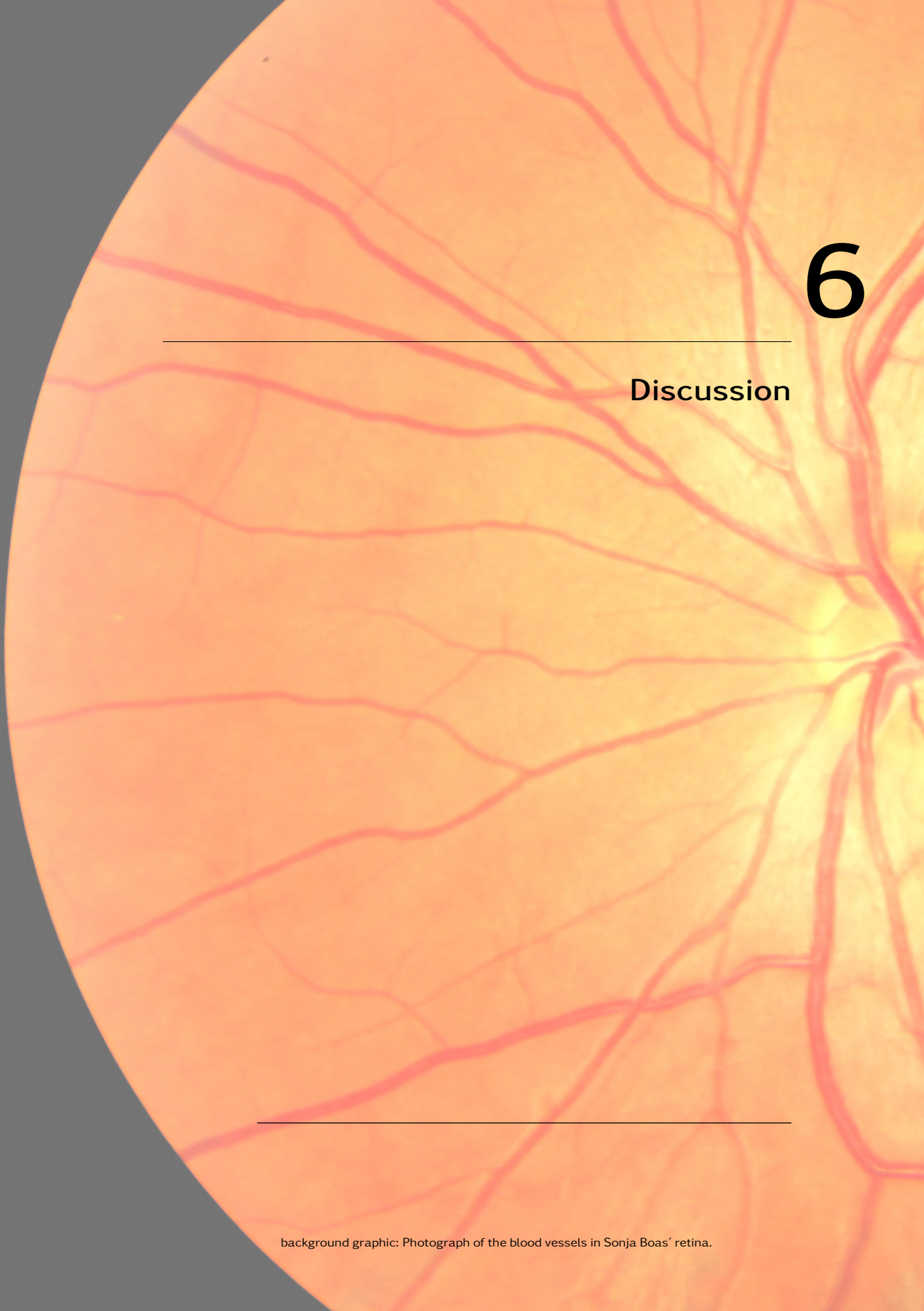
6

Discussion



background graphic: Photograph of the blood vessels in Sonja Boas' retina.

In this thesis, we have developed cell-based models to get a mechanistic understanding of the processes involved in angiogenesis. We have captured a number of key steps of angiogenesis. During angiogenesis, endothelial cells degrade the surrounding matrix (Chapter 2), while collective cell behavior in combination with intercellular signaling (Chapter 3) drives sprouting into the matrix. Once a sprout is formed, the sprout lumenizes to allow blood perfusion (Chapter 4). Each of these steps of angiogenesis are studied separately in this thesis and are compared to *in vitro* studies that focus on these steps. The steps were studied in isolation: such reduction of the biological complexity is required for a mechanistic understanding of the process. We believe that a model must be simple enough to explain the results, while complicated enough to generate results that initially surprise us. However, biology is more complex than we even dare to imagine; *in vivo*, all steps of angiogenesis are linked and intertwined. Therefore an important questions to ask is, can we link the isolated models of each step to get a better representation of the *in vivo* situation?

6.1 Interactions between matrix degradation and tip cell selection

In Chapter 2, we studied matrix degradation and invasion. For this purpose, we developed a cell-based computational model that represents an *in vitro* HMVEC-fibrin assay [38, 39] in which endothelial cells are seeded on a fibrin matrix and invade the matrix to form capillary-like tubule structures. It is unclear what mechanisms select the cells in the monolayer that initialize angiogenic ingrowth. The level of angiogenic ingrowth varies for different compositions of the fibrin matrix, it is higher on high molecular weight (HMW) fibrin than on low molecular weight (LMW) fibrin [39]. To ultimately understand what mechanisms cause angiogenic ingrowth, we used a computational model to study why ingrowth differs between HMW and LMW. Our model suggests that sprouting is driven by a feedback loop in which cells stimulate fibrinolysis, fibrinolysis releases TGFβ1 and TGFβ1 promotes cells to perform fibrinolysis. LMW has less binding sites than HMW [81] for proteins that link TGFβ1 to the matrix and keep TGFβ1 inactive. This results in a reduced availability of TGFβ1, consequently a lower activation of the feedback loop and in the end less ingrowth on LMW. Simulations show that due to the feedback loop, cells in the monolayer are naturally selected for ingrowth to lead sprouting. From experimental studies it is clear that intercellular signaling by Dll4-Notch, a process called tip cell selection, plays an important role in such a selection of leader cells [3]. In Chapter 3, we explicitly model Dll4-Notch signaling between cells. An interesting follow-up study would be to combine the local degradation feedback and lateral inhibition by Delta-Notch. Their

combination might affect the distances between ingrowth spots in the monolayer. Additionally, their combination might regulate sprout width. Sprouts produced in the fibrinolysis model are often wider than observed in experiments. Simulated sprouts are often spheroid shaped as cells neighboring the leading cell also sense the TGFβ1 feedback and then start to contribute to the invasion. Inclusion of tip cell selection in the plasmin-mediated fibrinolysis model might inhibit neighboring cells to react on the releases TGFβ1 by the leader cell, to allow formation of narrow sprouts. However, it is not know how the plasmin-mediated fibrinolysis, the TGFβ1 system and the Dll4-Notch signaling pathway interlink. Our models plasmin-mediated fibrin degradation and the tip cell selection can be coupled to study these interactions. This combined model could test different options for cross-regulation, e.g. the downregulation of uPAR by Notch signaling. By studying the effect of a variety of regulation pathways on the pattern of ingrowth spots in the monolayer and on sprout morphology, the model could give insights on the functionality of such cross-regulations.

In Chapter 3, we studied sprouting dynamics driven by collective cell behavior and intercellular signaling through Dll4-Notch. Initially it was thought that once a tip cell in a sprout was selected by the Dll4-Notch mechanism, that this cell remains the leader cell [3]. More recent experimental observations show competition of cells for the leading position [24, 25], a process called tip cell overtaking. We studied tip cell overtaking in two of our models of angiogenic sprouting [63, 64], in which sprouting is driven by contact-inhibited chemotaxis towards higher concentrations of a self-secreted growth factor [64] or by regular chemotaxis in combination with cell elongation [63]. We found that tip cell overtaking can result spontaneously from collective sprouting behavior in both models. Intercellular Dll4-Notch signaling can tune tip cell overtaking in simulations with a large difference in *Vegfr2* levels between cells. However, how realistic are such large differences in *Vegfr2* levels between cells *in vivo*? We propose that tip cell overtaking is a non-functional side effect of sprouting and that the function of VEGF-Dll4-Notch signaling might not be to regulate which cell ends up at the tip, but to assure that the cell that randomly ends up at the tip position acquires the tip cell phenotype. In the two models that we used to study tip cell overtaking, sprouts can freely invade the extracellular matrix without proteolytic degradation. It is likely that inclusion of degradation of the extracellular matrix and adhesion of cells to the matrix will affect the dynamics of collective cell behavior. For instance, cells at the flanks of sprouts might travel more efficiently past the sprout when aided by adhesion to the surrounding matrix. In addition, cells at the tip are slowed down because they first need to degrade the matrix for invasion. This delay of cell movement at the tip will likely affect the chemotaxis-driven sprouting mechanisms in the current tip cell overtaking models of Chapter 3.

For chemotaxis-driven sprouting, it is important that cells can quickly respond to changes in the curvature of the growth factor gradient. Thus, a delay of cell movement due to proteolysis of the matrix could affect the response to the growth factor and consequently the sprouting dynamics in these models. Since the matrix is present in *in vivo* sprouting, it is important to consider it in our models to bring them closer to the real situation. Simultaneously, this gives new opportunities for the validation of the sprouting mechanisms in our model. We should first validate if a combination of the fibrinolysis model and the tip cell selection model can still match the experimental data of tip cell overtaking. If not, this is an indication that sprouting might be driven by other mechanisms than that we assumed in our models and we can test if we can reproduce the experimental data by the use of different hypotheses for the mechanisms of sprouting. Mechanical cell-ECM interactions form such an alternative mechanism of sprouting [73, 74]. Cells are sensible to strains in collagen matrices and can also generate strains in the matrix themselves by pulling on it [73, 74]. Computational modeling by van Oers *et al*. [69] showed that cells form sprouts and organize into networks when they generate strain in the matrix and preferentially migrate towards higher strains in the matrix. A model by Santos-Oliveira *et al*. [197] shows the relation between sprout morphology and cell proliferation triggered by endothelial cell strain. In this model, a tip cell generate strain in the matrix and this tension produces strain and/or empty spaces, triggering cell proliferation in the following stalk cells to drive sprouting. The stress-stiffening response of fibrin matrices is extensively studied [198], as a next step it is interesting to study if mechanical-ECM interactions might also play a role in angiogenic fibrin invasion.

6.2 Lumen formation and dynamic sprouting

Once sprouts are formed, lumens form as a next step for the purpose of blood perfusion. In Chapter 4, we addressed the mechanisms of lumen formation. After decades of experimental research, two main hypotheses were formed to explain lumen formation. The first hypothesis is vacuolation [28, 29], in which fluid filled vesicles fuse into vacuoles that finally form a bridging tube through the cell or are secreted between cells to form extracellular lumens. The second hypothesis is cell-cell repulsion [32, 33] that suggests that negatively charged ions on adjacent cell membranes repulse one other to initiate lumen formation and cell shape changes further open the lumen. Our model of Chapter 4 shows that lumens can form for both hypotheses for small parameter ranges, but their combination is far more robust to changes in the parameter settings, suggesting synergy of the two hypotheses. The contradicting experimental observations might be explained by the vessel sizes in different studies; lumen formation looks like vacuolation in simulations with

one-cell thick vessels, whereas it looks like cell-cell repulsion in multi-cellular vessels. A combination of the two hypotheses might make lumen formation more robust to changes in the environmental settings, such as different vessel sizes. In line with our modeling results, recent experimental work indicates that both hypotheses contribute to lumen formation in intersegmental vessels (ISV) of zebrafish [199]. Single cell analysis, by fluorescently labeling the cell nuclei and cell membranes simultaneously, demonstrated the coexistence of intercellular lumens within single cells and extracellular, multicellular enclosed lumens [199].

Robustness of lumen formation to changes in the environment due to synergy of vacuolation and cell-cell repulsion could especially be relevant when lumen formation is already initiated during sprouting, rather than after the sprout is formed. To study this, we made a preliminary model of cells seeded on a bead from which sprouts dynamically form by contact-mediated inhibition of chemotaxis [64]. Lumens form during sprouting by a combination of the vacuolation and the cell-cell repulsion hypotheses (Figure 6.1). In this simulation, lumens form robustly, but they keep on growing by the transfer of fluid by the vacuoles from the extracellular environment towards the interior lumen. Interestingly the vacuolation hypothesis assumes that the vessels are sealed through strong cell-cell interactions and cannot leak fluid [29], whereas the cell-cell repulsion hypothesis assumes vessels are leaky with paracellular openings [32]. This could experimentally be tested by the injection of a dye in the luminal space. The vessel is sealed if the dye remains within the lumen, and is leaky if it diffuses to the extracellular medium. The vessels are sealed in the current model, and we could use the model to hypothesize how the volume of the lumen is controlled in this case. A possible mechanism would be a negative feedback of luminal pressure on the uptake of extracellular fluid from the extracellular matrix. Alternatively, we could adapt our model to include leaky cells. In that case, it is interesting to study what mechanism prevent lumens to collapse in absence of the luminal pressure. Possibly, adhesion of cells to the surrounding matrix suffices. In this case, proteolytic degradation of the surrounding matrix is likely to affect the size of the lumen, indicating that this research question might also require the modeling of matrix degradation. Thus, combining computational models leads us to new research questions that allow for further study and comparison of the two lumen formation hypotheses.

6.3 Model sensitivity analysis

Altogether, it seems that to combine the well-studied isolated models is a good method to generate new research questions and to represent the *in vivo* situation more closely. However, caution should be taken when combining

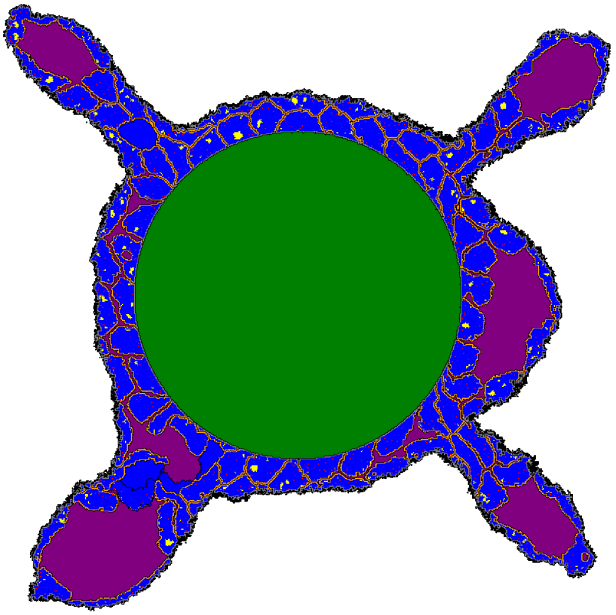


Figure 6.1: Dynamic lumen formation during sprouting. Endothelial cells (blue) are seeded on a bead (green) and polarize their membranes into apical (orange) and basolateral (gray) membranes. Subsequently, sprouts form through contact-mediated inhibition of chemotaxis. During sprouting, lumenal fluid (purple) is created by the secretion of vacuoles (yellow) and, in addition, lumens are created through cell-cell repulsion.

models. It results in large and complicated models with many parameters. As a result, analyzing the influence of these parameters in the model becomes more challenging. One-or two-dimensional parameter sweeps can still give insights, but it is important to realize that this is a reflection of a local space in the output distribution as the other parameters are fixed at a nominal value. A global sensitivity analysis can rank the impact of multiple parameters at the same time on the variance of the output. Importantly, it can also identify the impact of parameter interactions. In Chapter 5, we discussed how one can apply a global sensitivity analysis on stochastic, multi-factorial models, such as the cellular Potts models in this thesis. A simple model of vascular morphogenesis, using contact-mediated inhibition of chemotaxis for sprouting [64] (contact inhibition model), is used as a case study. The chapter introduces a flowchart to perform a global sensitivity analysis and focuses on the pitfalls and on the reliability of the results. We developed a rule to predict *a priori* how many simulations are required to get reliable results. One of the pitfalls to consider in a global sensitivity analysis is that the results might not represent the answer to the question at hand. This is strongly de-

pendent of the parameter ranges and output measures one chooses to study the problem. For example, a parameter could have a very large influence, but solely on an interval of, say, 1 to 10. A global sensitivity analysis would identify this parameter as important for a chosen interval of 1 to 10, but not for an chosen interval of 1 to 1000, thus it is crucial to carefully match the choice of the parameter intervals to the regions of interest for the question. Additionally, different output measures can give different global sensitivity analysis results. For instance, the analysis in Chapter 5 for the contact inhibition model showed that compactness of networks depends on a combination of model parameters, whereas the diffusion coefficient of the self-secreted chemoattractant has a dominant impact on the lacuna count.

6.4 Three-dimensional models

In some conditions, angiogenesis can be considered as a quasi-two-dimensional process, such as angiogenesis in the retina *in vivo* and endothelial network formation on cover slips *in vitro*. For this reason, it is reasonable to model angiogenesis as a two-dimensional process. In addition, two-dimensional simulations are faster to compute and easier to analyze than three-dimensional simulations. However, even in quasi-two-dimensional angiogenesis, the third dimension could be of some influence. For instance, the interaction surface between neighboring cells for intercellular signaling can still locally change by the third dimension. Another clear example in which the third dimension could be important is lumen formation. Lumen formation by the vacuolation hypothesis suggests that fluid filled vacuoles fuse in the interior of the cell and finally form a tube through the entire cell that fuses to the to the luminal tubes in the cell in front and behind it in the sprout. The cell becomes a hollow cylinder in three-dimensions, but a cell will appear to be split up in two pieces by the lumen in a two-dimensional cross-section along the long axis. Fortunately, all of our models are scalable to three-dimensions, because all the cells obey local rules. We believe that cells are not preprogrammed during angiogenesis, but are guided by the information from their local surroundings, e.g. by the pericellular gradient of chemoattractant or by the local concentration of fibrin. Therefore, each rule for the cells in our models depends only on the local environment of cells and can consequently be extrapolated from two-dimensions to three-dimensions. As an illustration, Figure 6.2 shows a preliminary three-dimensional model of a sprout that invades fibrin and forms a lumen inside. In summary, future steps can be taken to represent *in vivo* models more closely by combining models as well as by extending them to three-dimensions. The better a model matches reality, the more likely that the insight that are gained from it are directly interpretable and useful for the *in vivo* situation. In addition, a more detailed model can

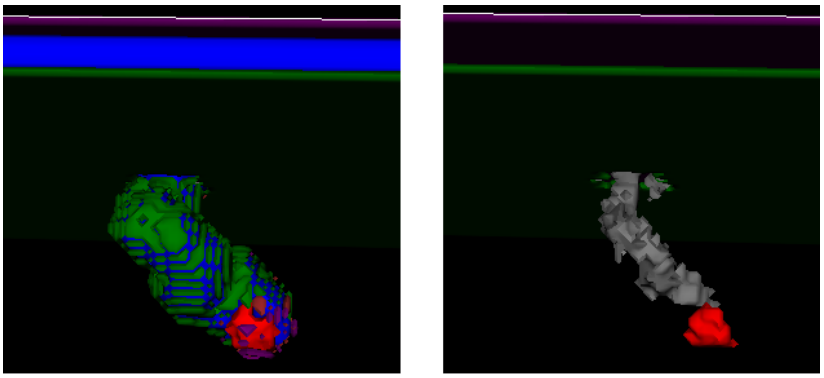


Figure 6.2: Sprouting and lumen formation in three-dimensions. Endothelial cells (blue) are seeded on an extracellular matrix (green). One cell in the monolayer is selected as a tip cell (red). The tip cell secretes proteolytic enzymes to form a tunnel into the matrix for sprouting. On the left, an image is shown in which a sprout is formed inside the matrix and the matrix is made transparent for visualization. A lumen is formed inside the sprout (gray material in image on the right), simply by creating vacuoles that migrate to the center of the sprout and then fuse into one single lumen.

benefit the validation of the model, because it is does not suffer from layers of abstraction that could make it difficult to link the effects of parameter changes in the model to the *in vivo* effects. However, it is important to keep in mind that the goal of modeling is to understand a biological process, not to mimic it, thus caution should be taken that the model does not become to complex to mechanistically understand its results.

6.5 Model validation

Validation of our models to experiments performed in the laboratory is crucial. We mostly compare results to *in vitro* models as these are more controlled and isolated, similar to our *in silico* studies. The control of e.g. the composition of the extracellular matrix, the addition of growth factors, and the inhibition of protein activity or gene expression allows for manipulation of the cell behaviors and properties. As a validation of *in silico* models, we try to reproduce such *in vitro* manipulation experiments. For this purpose, ideally, all model parameters are directly coupled to such controllable experimental factors, e.g. a specific protein. Unfortunately, this is not always true in practice. Fortunately, some parameters can be coupled in a qualitative matter. For instance, protein concentrations can be tuned relative to each other. Dose-dependent experiments for manipulation of cell adhesion strengths, rates (e.g. pinocytosis rate) and signaling pathways can help to

tune the representing qualitative parameters in the model. Additionally, various parameters could be quantified experimentally to allow for quantitative model predictions. The adhesion strength of cells can be quantified by the force that is required to pull them apart [161]. This method might be useful to find quantitative values, or at least the relative ordering, of the contact energy parameters that describe adhesions between polarized cells, non-polarized cells and the extracellular matrix.

6.6 Relevance of modeling

What can the Life Sciences community learn from modeling? In the first place, computational models are very useful to test hypotheses. In comparison with experimental assays, a large benefit of computational models is that you can define the level of complexity. Often, we like to study a specific mechanism or regulation system in isolation, such as matrix degradation (Chapter 2), tip cell overtaking (Chapter 3) or lumen formation (Chapter 4). In a computational model, one can focus on the main components and interactions of the system of interest and is not hindered by all the proteins, pathways and environmental conditions that interfere with the system *in vivo*. Sometimes, models show that what was hypothesized to be enough to drive a system is not sufficient to reproduce experimental data. This indicates that the assumptions and mechanisms of the model should be reevaluated. Alternatively, abstraction of a complex mechanisms to a simple model can show that biological phenomena can be much simpler than initially thought. For instance, organization of the axial body segments in vertebrates involves many genes (HOX genes) and regulating pathways [200, 201]. However, patterning might be driven by a far more simple mechanism in some other cases. Mathematical modeling showed that patterns, such as stripes and spots, can emerge through self-organization by a simple reaction-diffusion system [105], in which an activator activates itself and activates an inhibitor that diffuses slower than the activator. Experimental and mathematical modeling results indicate that such a mechanism possibly controls digit patterning, in which the dose of *Hox* genes modulates the digit period or wavelength [202]. In addition, a reaction-diffusion mechanism involving WNT and its inhibitor DKK might regulate spacing of epidermal hair follicles [203].

When we feel that we understand the isolated process, we can start to combine model components to closer match the experimental settings. Some examples of model combinations are discussed in the previous sections, e.g. a combination of matrix degradation and tip cell overtaking. Extending the complexity of models can lead to new research questions and interesting new insights. The greatest strength of modeling is that it allows us to test many different hypotheses in a cheap and efficient manner. Each hypothesis could

result in a different outcome of the model. Predictions on the model outcome emerge on a higher level from modeling hypotheses on a lower level. For instance, lumen formation or tip cell overtaking on a tissue scale arise from assumptions on cellular and intercellular interactions. By testing the model predictions from different hypotheses with experiments, hypotheses can be validated or falsified. As a first validation, we test our model results with readily published data. In this thesis, we for instance compared the tip cell overtaking rates with published experimental data [24, 25]. The next step is to experimentally verify new, experimentally unexplored model predictions that give new biological insights. As an example, the lumen formation model predicted that vacuolation and cell-cell repulsion function synergistically and this hypothesis was recently validated experimentally by Yu *et al.* [199]. In addition, we have a close cooperation with a laboratory specialized in fibrin invasion with whom we plan to test the hypothesis from Chapter 2 that a uPAR-plasmin-TGFβ1 positive feedback loop drives sprouting in their experimental setup. Validation of model predictions is essential for the acceptance of new hypotheses. A model can merely make a strong case that a hypothesis is likely correct. Only by experimental validation, one can be confident that a hypothesis actually describes what is happening *in vivo*. This is why we cannot stress enough how important a close cooperation between computational and experimental biologists is to make steps in the Life Science field.

6.7 Model predictions

By studying the computational models in this thesis, we were able to suggest some experiments to test our model predictions. Here we sum the four most important predictions from the models in this thesis that require experimental validation:

1. The probability of angiogenic ingrowth in a monolayer of endothelial cells in the *in vitro* HMVEC-fibrin assay [38] depends on a TGFβ1-fibrinolysis feedback loop. There is a reduced angiogenic ingrowth on LMW compared to HMW as a result of lower levels of latent-TGFβ1 bound to LMW. Our model predict that addition of TGFβ1 antibodies to cells cultured on HMW should reduce sprouting.
2. Tip cell overtaking occurs spontaneously during sprouting by the cell mixing induced by the collective cell behaviors driving sprouting in our models. In the contact inhibition model, cells are pushed forward in the center of the sprout by the sprouting force and move backward along the sides of the sprouts driven by the gradient of the chemoattractant towards the spheroid center. This suggests that tracking of the position of cells in the sprout in experiments can give information on the driving

mechanisms of cell-mixing in sprouting. This experiment has already been done [25], but the experimental data is not publicly available.

3. Lumen formation is most efficient and robust for changes in the parameters and in the environment when vacuolation and cell-cell repulsion function synergistically. Our model results predict that inhibition of vacuolation by reducing pinocytosis should prevent lumen formation in capillaries, but not in larger vessels, whereas inhibition of cell-cell repulsion by cleavage of negatively charged extracellular proteins should destabilize lumen formation in all vessel types. Recent experimental work in ISV of zebrafish [199] supports our hypothesis that vacuolation and cell-cell repulsion both contribute to lumen formation.
4. A global sensitivity analysis for the contact inhibition model showed that the diffusion coefficient alone is dominant for variation in the lacuna count of vascular networks. The number of lacuna is large for small values of the diffusion coefficient, whereas no lacunae are formed for large values of the diffusion coefficient. To reach such small diffusion coefficients, the chemical in question needs to have a strong binding interaction with the extracellular matrix. Our model suggests VEGF as a possible candidate for the chemoattractant, but the attractive force might be mediated by another chemoattractant (e.g. CXCL12 [64]). Therefore, we suggest to experiment with different matrix types and different binding-epitoop blockers, to see if it effects the lacuna count, and if so, to trace back the responsible chemoattractant by checking which chemical corresponds to the most influencing set of changes in binding-epitoops.

In summary, we developed computational models to address key steps in the complex process of angiogenesis. Each model gave new insights in the mechanisms of the isolated steps. Future work can combine and intertwine these models to come to a closer representation of *in vivo* angiogenesis. Experimental validation of the model predictions is essential to test the validity of our model assumptions and hypotheses. Thus, by a combination of computational modeling and experimental assays we are unraveling the mechanisms of angiogenesis, step by step.