Timm Schroeder

CURRICULUM VITAE

Name:	Timm Schroeder born 3. September 1970 in Pretoria, South Africa German, married
Languages:	man: German, English, French, Japanese, Latin machine: VB, VB.NET, VBA, LUA, C++
Position:	Professor of Cell Systems Dynamics, Department of Biosystems Science and Engineering, ETH Zürich, Mattenstr. 26, 4058 Basel, Switzerland timm.schroeder@bsse.ethz.ch

Career

2013–	**Full Professor, Cell Systems Dynamics** Department of Biosystems Science and Engineering **(D-BSSE)** Swiss Federal Institute of Technology **(ETH) Zürich,** Basel, Switzerland 2015–17/2017–: Deputy-/**Head of Department** D-BSSE
2011–13	Director, research unit Stem Cell Dynamics, **Helmholtz Zentrum München** – German Research Center for Environmental Health, Neuherberg, Germany
2004–11	Principal Investigator (tenured 2009), Research group «Hematopoiesis», Institute of Stem Cell Research, **Helmholtz Zentrum München** 2006–13: Deputy Director, Institute of Stem Cell Research
2002–04	Visiting Researcher, Group for Stem Cell Biology, **RIKEN Center for Developmental Biology,** Kobe, Japan
2000–02	PostDoc, **GSF, Munich,** Germany 2001 and 2002: Research visits in Kyoto (groups Tasuku Honjo and Shinichi Nishikawa) and Harvard (group Dan Tenen) Universities, Japan and USA

1997–2000	Dr. rer. nat. thesis *(summa cum laude 2001),* Chair of Genetics, Friedrich Alexander **University Erlangen** and Institute for Clinical Molecular Biology and Tumour Genetics, **GSF, Munich,** Germany 1999 and 2000 Research visits, Kyoto University, Japan
1991–97	Studies in Biology, Friedrich Alexander **University Erlangen,** Germany
1990-91	Mandatory civilian service, research technician in cancer research, University hospital Grosshadern, Munich, Germany

Research awards

2018	Cloëtta Prize, Professor Dr. Max Cloëtta Foundation, Switzerland
2017	Erwin Schrödinger Prize – The Stifterverband Science Award for interdisciplinary research by the German Helmholtz Association, shared with Drs. Theis, Marr (Germany) and Haghverdi (UK)
2012	McCulloch&Till Award, International Society for Hematology and Stem Cells
2002	GSF Ph.D. student of the year award
2001	New Investigator Award, International Society for Experimental Hematology

- **Selected committee memberships**
- International Society for Experimental Hematology (ISEH): Vice President/President Elect/**President/**Immediate Past President and Executive Committee. 2014–18
- Personalized Health Allianz Zürich - Basel Governing Board. 2017-
- European Hematology Association (EHA) Research Committee 2015–
- Swiss Stem Cell Network (SSCN) Steering Committee 2014–
- Basel Stem Cell Network (BSCN), Switzerland Steering Committee 2013–

SELECTED PUBLICATIONS

1. Coutu DL*, Kokkaliaris KD, Kunz L and **Schroeder T* (2018).** Multicolor quantitative confocal imaging cytometry. ***Nature Methods*** 15, 39–46.

2. Coutu DL, Kokkaliaris KD, Kunz L and **Schroeder T (2017).** Three-dimensional map of nonhematopoietic bone and bone-marrow cells and molecules. ***Nature Biotechnology*** 35, 1202–1210.

3. Hoppe PS, Schwarzfischer M, Loeffler D, Kokkaliaris KD, Hilsenbeck O, Moritz N, Endele M, Filipczyk A, Gambardella A, Ahmed N, Etzrodt M, Coutu DL, Rieger MA, Marr C, Strasser MK, Schauberger B, Burtscher I, Ermakova O, Bürger A, Lickert H, Nerlov C, Theis FJ and **Schroeder T (2016).** Early myeloid lineage choice is not initiated by random PU.1 to GATA1 protein ratios. ***Nature*** 535, 299–302.

4. Hilsenbeck O, Schwarzfischer M, Skylaki S, Schauberger B, Hoppe PS, Loeffler D, Kokkaliaris KD, Hastreiter S, Skylaki E, Filipczyk A, Strasser M, Buggenthin F, Feigelman JS, Krumsiek J, van den Berg AJJ, Endele M, Etzrodt M, Marr C, Theis FJ* and **Schroeder T* (2016).** Software tools for single-cell tracking and quantification of cellular and molecular properties. ***Nature Biotechnology*** 34, 703–706.

5. Filipczyk A, Marr C, Hastreiter S, Feigelman J, Schwarzfischer M, Hoppe PS, Loeffler D, Kokkaliaris KD, Endele M, Schauberger B, Hilsenbeck O, Skylaki S, Hasenauer J, Anastassiadis K, Theis FJ* and **Schroeder T* (2015).** Network plasticity of pluripotency transcription factors in embryonic stem cells. ***Nature Cell Biology*** 17, 1235–1246.

6. Filipczyk A, Gkatzis K, Fu J, Hoppe PS, Lickert H, Anastassiadis K* and **Schroeder T* (2013).** Biallelic expression of nanog protein in mouse embryonic stem cells. ***Cell Stem Cell*** 13, 12–13.

7. Eilken HM, Nishikawa S-I and **Schroeder T (2009).** Continuous single-cell imaging of blood generation from haemogenic endothelium. ***Nature*** 457, 896–900.

8. Rieger MA, Hoppe PS, Smejkal BM, Eitelhuber AC and **Schroeder T (2009).** Hematopoietic cytokines can instruct lineage choice. ***Science*** 325, 217–218.

9. **Schroeder T (2008).** Imaging stem-cell-driven regeneration in mammals. ***Nature*** 453, 345–351.

10. **Schroeder T (2005).** Tracking hematopoiesis at the single cell level. ***Annals of the New York Academy of Sciences*** 1044, 201–209.

MOLECULAR STEM CELL FATE CONTROL: QUANTIFICATION OF CELLULAR AND MOLECULAR DYNAMICS AT THE SINGLE-CELL LEVEL

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Summary

Despite decades-long intensive research, surprisingly many long-standing questions in stem cell research remain disputed. One major reason is the fact that we usually analyze only populations of cells, rather than individual cells, and at very few time points of an experiment, rather than continuously. My group therefore develops imaging systems including the required software to long-term image, segment and track individual cells, and to quantify e.g. divisional history, position, interaction, and protein expression or activity of all observed individual cells over many generations. Dedicated software, machine learning and computational modeling enable data acquisition, curation and analysis. Custom-made microfluidics devices improve cell handling, observation, dynamic manipulation and molecular analysis. The resulting continuous single-cell data is used for analyzing the dynamics, interplay and functions of signaling pathway and transcription factor networks in controlling hematopoietic, pluripotent, skeletal and neural stem cell fate decisions. After the first 1.5 decades of my independent research group, I here review these technological developments, and some of the long-standing biological questions in stem and progenitor cell biology they have contributed to answer.

Introduction: The need for long-term single-cell quantification of cellular and molecular dynamics

How do cells behave to generate and regenerate healthy tissues? What has changed in disease? How do molecular machineries control these cell behaviors, and how can we manipulate them to control cell fates for therapy? These questions are at the core of most biological and biomedical

research projects. However, as discussed for some examples below, surprisingly many obvious questions remain without satisfying and accepted answers despite decades of intensive research. This is certainly the case in hematopoietic stem cell biology, the classical mammalian stem cell system. Many of the concepts, terms and questions of stem cell research have been defined in this system since the middle of last century, but remain unresolved controversies. During my doctoral thesis, where I worked on the possible effects of Notch activation on hematopoietic progenitor cell fates, I realized that many basic conceptual questions in the field remain disputed. I felt that the lack of adequate technologies for quantifying the dynamics of cellular and molecular behaviors is one important reason for this lack of satisfying answers.

Our blood system produces millions of cells every second of our life. As in other cell systems, the number and type of cells produced must be tightly regulated, and also adapted over time to changing needs. Failure to produce the right number of the right cells at the right time and location can quickly lead to deadly diseases like anemia or leukemia. It seems obvious that the first step in analyzing the molecular control of the underlying cell fate choices (Fig. 1) must be to know what cells actually do in health and disease, and how cell fate choices change upon molecular mutation and manipulation.

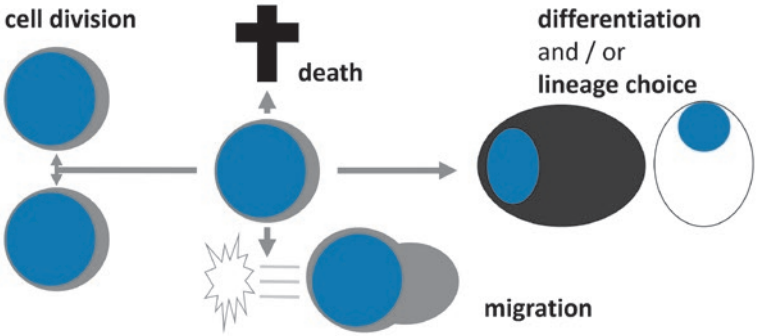

Figure 1: Cell fate options of multipotent stem and progenitor cells. *These are chosen in close reciprocal dynamic interaction with the microenvironment of individual cells.*

However, surprisingly, we typically lack the precise knowledge about what our cells of interest really do. This is because cell and molecular behavior is usually analyzed as population averages and / or by snapshot analyses – and not continuously at the single-cell level. This snapshot average approach is dangerous, since it will mask properties and behaviors of individual cells, and typically leaves too much room for interpretation when generating conclusions from primary data (Schroeder, Annals Of The New York Academy Of Sciences 2005; Nature 2008; Nature Methods 2011; Etzrodt et al., Cell Stem Cell 2014; Hoppe et al., Nature Cell Biology 2014; Skylaki et al., Nature Biotechnology 2016). While the need for single cell analyses has long been recognized, and commercial off-the-shelf solutions like FACS, colony assays and more recently single-cell sequencing are increasingly available and used, the continuous live single-cell quantification of dynamics remains challenging and missing in most studies. As illustrated in Figure 2, even simple questions like *"How did one cell generate four cells?"* allow many competing interpretations about the underlying cell fate choices, even when analyzed at the single-cell level but with data only from the start and end of the experiment (Fig. 2).

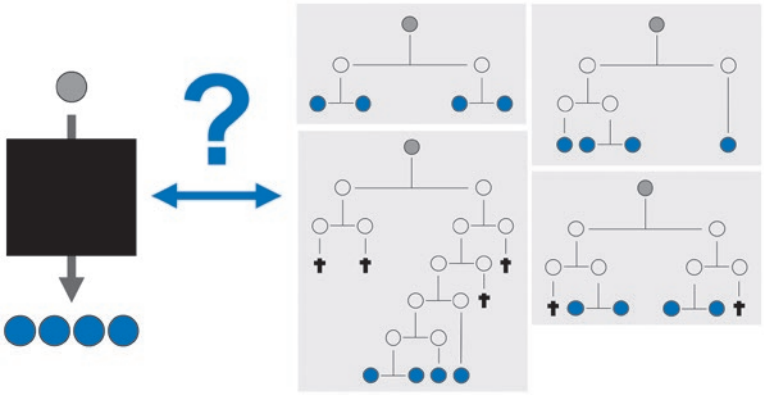

Figure 2: Continuous single-cell fate quantification is required to understand the cellular dynamics underlying normal and pathological phenotypes.

As shown in this example, snapshot data allows very different assumptions about the involved cell fate choices which are all compatible with the measured data. For example, half of the cells might have died, or none of the cells could have died. Obviously, the assumed reasons e.g. for normal or pathological tissue (re)generation, possible interventions in disease, and the molecular machineries picked to the analyze for many years would fundamentally differ depending on whether one picks the first or second (of many possible additional) interpretation.

The same not only holds true for quantifying cell fates, but also for analyzing the dynamics of the molecules involved in their control. Depending on how frequent and long the e.g. expression, activation or subcellular location of molecules of interest is analyzed, one will come to very different conclusions about possible dynamics like oscillations, step functions or transient peaks. However, this knowledge is crucial to understand how the molecular machineries leading to normal or diseased cell behavior are wired and implemented, and how to manipulate them for therapy. Again, typical snapshot average data is too ambiguous and usually allows different competing interpretations (Schroeder, Nature Methods 2011; Etzrodt et al., Cell Stem Cell 2014; Skylaki et al., Nature Biotechnology 2016).

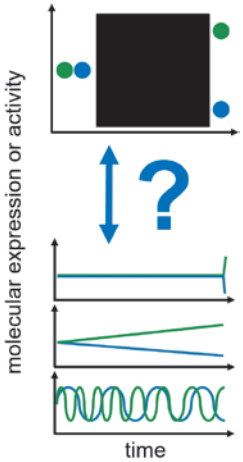

Figure 3: Continuous single-cell molecular quantification is required to understand the molecular dynamics underlying cell fate control.

Continuous single-cell quantification of molecular dynamics is therefore essential. This often requires much higher frequencies of image acquisition to temporally resolve fast molecular dynamics, posing even bigger technical challenges to data acquisition and analysis. This would be balanced by shorter required imaging durations, since the molecular events are much shorter than cell fate choices – minutes to hours versus days. However, since the functional relevance of specific molecular dynamics in individual cells only becomes clear when being able to link them to the future cell fate decisions of the same cell or its progeny, the combination of both the high-frequency shorter molecular imaging at the beginning of the experiment, and the following lower-frequency long-term cell fate imaging over days is required.

With this comprehensive novel kind of data, the confusing heterogeneous effect of e.g. signaling inputs on cell fate choices of individual cells, or possibly on the same cell with changing intracellular molecular states over time (Fig. 4) can likely be better understood.

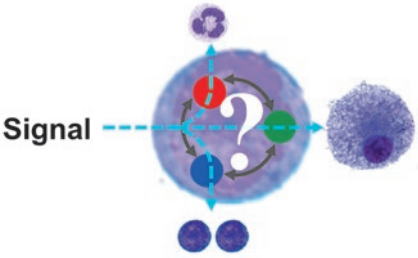

Figure 4: Changing or cycling intracellular molecular states, e.g. due to cell cycle progression, could lead to changed modulation of signaling inputs and thus altered or cycling effects on cell fate of the same signaling pathway in the same cell over time.

In conclusion, quantification of cell fate choices and molecular dynamics at the single-cell level and continuously over time is essential for a precise understanding of the cellular and molecular mechanisms underlying health and disease. Here, I will discuss some of the technologies developed in my group to enable these quantifications, and how we used them to try to answer some of the long-standing disputes in the field.

The purpose of this manuscript is the review of some of my own group's work honored with the Cloëtta Prize, not a comprehensive review of the literature. The listed references are therefore restricted to my own publications. For a more comprehensive and balanced representation of the relevant literature, I refer to the references in my listed publications.

Development of technologies for long-term single-cell quantification of cellular and molecular dynamics

For the reasons discussed above, we develop novel technologies allowing the continuous long-term imaging, single-cell tracking and quantification of cells.

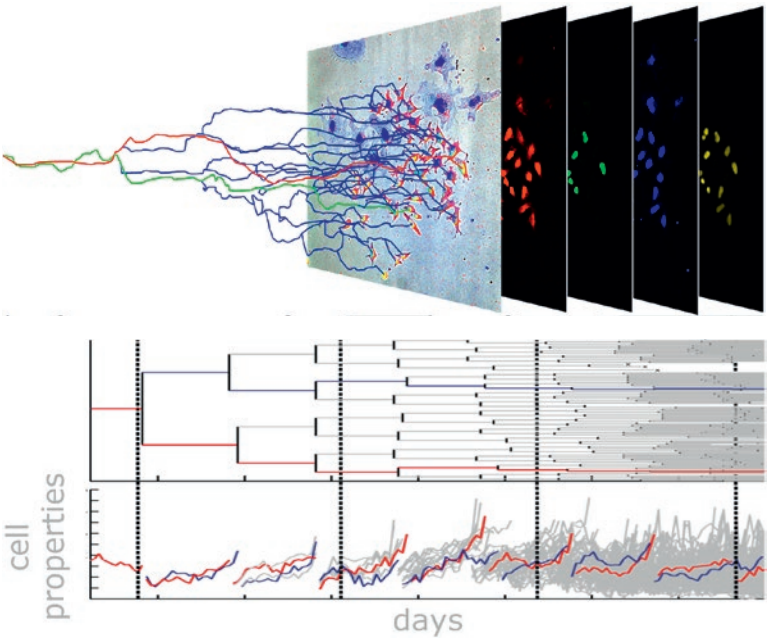

Figure 5: Long-term imaging, segmentation and tracking enables the single-cell quantification of cellular and molecular dynamics over up to weeks. *Adapted from (Hilsenbeck et al., Nature Biotechnology 2016; Skylaki et al., Nature Biotechnology 2016).*

Mammalian cells, including rare primary stem and progenitor cells, are purified, cultured, manipulated and observed by time-lapse video microscopy over up to weeks. The mobility of cells requires high temporal imaging frequency to prevent the confounding of cell identities when tracking individual cells. This requirement of reliably taking frequent pictures over long periods of time brings many technological challenges. The imaging hardware has to be much more robust and reliable than for normal imaging experiments. We typically take around one picture per second, which means that mechanical parts like shutters "click" one million times in less than two weeks, and thus sometimes even within one single experiment. The typical guaranteed hardware cycle times for two-year warrantee periods are thus used within days to weeks. Mechanic wear and tear are not the only problem. Given that we have to observe our cells of interest with high temporal resolution to not lose track of their identities, failures of acquiring even individual pictures can render a movie of 10 000s of pictures useless. While failing e.g. every 100th image acquisition when manually taking pictures is not problematic – one can just click a button again – it is catastrophic for high frequency time-lapse imaging where it would lead to loss of every single experiment conducted. However, while these are challenging problems, they can be solved with the right combination of (usually not off-the-shelf) commercially available hardware.

The biggest challenge is data processing, storage and analysis. Not only are the shear amounts of data scary. The imaging capacity in my laboratory can currently produce about one petabyte of primary data per month. Just the storage (not analysis) of this one month worth of data on the cheapest storage hardware available in academic IT service departments will cost more per year every year than typical research grants pay for annual consumables of individual research projects. More importantly, both, the reliable and efficient acquisition, and the meaningful and statistically sound analysis of this kind and volume and data remains impossible with commercially available software. Still in 2018, and certainly in the early 2000s when I started working on these challenges. Commercially available custom software by reputable software companies for cell tracking in time-lapse data existed then and looked promising. However, after wasting 10 000s of US dollars – my apologies to my

Japanese mentor Shinichi Nishikawa – it turned out that in routine day-to-day use, it was neither a match for the data volumes at hand, nor for the required usability, reliability and specific functionality.

Figure 6: Software tools for single-cell segmentation (fasTER), tracking (tTt) and quantification (qTfy, XiT) developed in the Schroeder group. *All our published software is open-sourced and can be downloaded at www.bsse.ethz.ch/csd/software.html.*

I therefore had to start programming myself, the result of which (tTt, Fig. 6) (Hilsenbeck et al., Nature Biotechnology 2016) has meanwhile been much further developed by contributions of many, and proven useful for many published and ongoing studies in groups on 4 continents. Together with self-programmed software for microscope hardware control, computer vision and machine learning for cell recognition and segmentation (Hilsenbeck et al., Bioinformatics 2017), automated cell tracking, image correction (Schwarzfischer et al., Proceedings Microscopic Image Analysis With Applications In Biology 2011; Buggenthin et al., BMC Bioinformatics 2013; Peng et al., Nature Communications 2017) and quantification (Hilsenbeck et al., Nature Biotechnology 2016), as well as statistical analysis of pedigree structures (Stadler et al., Journal Of Theoretical Biology 2018) and machine learning for high-dimensional pattern recognition and cell fate predictions (Buggenthin et al., Nature Methods 2017), it is now part of a continuously growing software pipeline.

This pipeline enables the long-required continuous long-term single-cell quantification of many dimensions of cellular and molecular properties, dynamics and kinship. For example, divisional history, position, interaction, and protein expression or activity are recorded and quantified for all observed individual cells over many generations (Fig. 5). As discussed above, this is a crucial prerequisite for the improved understanding of

molecular cell fate control (Eilken et al., Nature 2009; Rieger et al., Science 2009; Filipczyk et al., Nature Cell Biology 2015; Hoppe et al., Nature 2016; Kokkaliaris et al., Blood 2016).

Software is a crucial, but not the only required technology required to custom-develop for this approach to work efficiently. All components of a single-cell tracking experiment must be perfectly adjusted to each other, from the cells and molecular reporter and manipulation materials used, over the culture and liquid handling systems at hand, the imaging hard- and software to the data acquisition, storage and analysis pipeline. All these components will have to be adjusted with each new biological question to be analyzed, a process often requiring many iterations of optimizations. One important missing part for us was the lack of commercially available custom micro- or macro-fluidic devices optimized for efficient liquid handling, cell culture and imaging. With the help of our engineering colleagues and facility at the Department of Biosystems Science and Engineering of the ETH Zurich in Basel, we have therefore begun to develop and produce our own custom-made components addressing the specific experimental needs of different biological systems analyzed (Fig.7).

Figure 7: The flow from computationally aided design of microfluidic chip to the produced real-world device. *Adapted from (Dettinger et al., Analytical Chemistry 2018).*

These combined solutions routinely and robustly work in my and other groups. However, they still require dedicated specialists who understand the value of the new kind of generated data to be willing to invest their time. Many challenges remain, and generic one-fits-all solutions do not exist. My disappointing answer to the typical question of interested colleagues "How do I best do these experiments" unfortunately remains (and will likely also remain in many instances in the future) "It depends". Each novel combination of specific biological question, available biological material, reporters and culture system, required imaging frequency, dimensionality and duration, and optical properties of the observed structures will typically need rounds of optimization, and specialists' love and care in acquisition and analysis of data (Skylaki et al., Nature Biotechnology 2016). In many cases, automation of data analysis fails due to the lack of e.g. reliable computer vision solutions, and manual curation, error correction or even analysis and generation remain required. Given the new kind of continuous single-cell quantification and kinship data, the required mathematical tools often have not yet even been developed, let alone implemented into easy to use automated software tools, and a lot of groundwork is still required in this area.

Nevertheless, I am convinced that quantification of behaviors over time, as opposed to states at one timepoint, will be the future also of routine screening approaches e.g. in pharmaceutical industry. Indeed, we have begun to use long-term singe-cell fate quantification for mid-throughput screening for novel extracellular regulators of stem cell self-renewal expressed by their niche. By observing the behavior of individual stem cells in complex co-cultures with stromal cells, and the concurrent manipulation of 50 different candidate genes in the stem cells' environment, we were able to identify more novel regulators in a year than the field had in the previous 20 years of research using the same cell models (Kokkaliaris et al., Blood 2016). This well demonstrates that the conclusions yielded by this continuous observation approach are typically so much more robust and allow insights which would otherwise be missed, that higher investments into the more demanding technological approach will quickly pay off. In particular for recurring problems as in high-throughput screening with standardized cells and questions to be analyzed, the relevant steps can be automated with sufficient reliability. Most of the

current challenges in this area are simply engineering problems which can be solved with sufficient time and money.

Finally, in addition to the single-cell tracking approaches of cultured cells, we have started to also optimize quantitative high-dimensional large-volume 3D imaging in vivo. Through optimization of existing, and development of novel materials, protocols and custom software, large volumes of tissues, e.g. over the total length and width of a full mouse femur in thick sections, can now be imaged with sub-cellular resolution in up to 10 colors and quantitatively analyzed (Fig. 8).

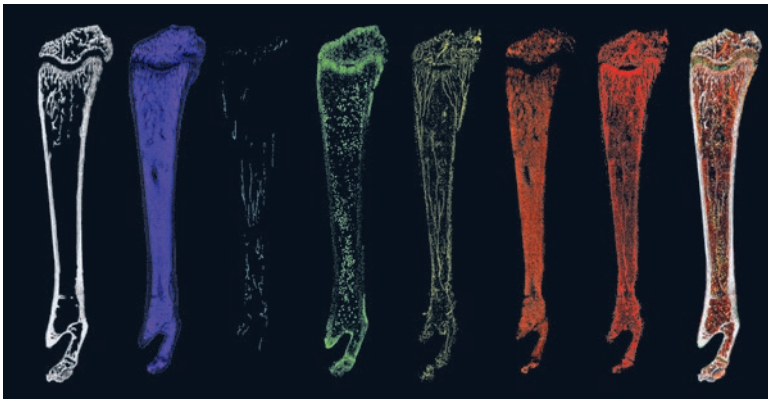

Figure 8: Large-volume multi-color 3D imaging of bone and bone marrow. *A thick section of a full mouse femur imaged for different molecular and cellular components in seven colors is shown (Kunz, Coutu, Kokkaliaris and Schroeder, unpublished).*

Importantly, we developed these approaches for use on standard confocal microscopes available in many research institutions and with precise description of the relevant individual steps to help democratizing large tissue quantitative multicolor cytometry.

Along the same line, all data acquired during development of this technology with a lot of effort, testing hundreds of expensive antibodies, and imaging hundreds of bones over thousands of microscopy hours, was made openly available for the community (Fig. 9) to freely download and use (Coutu et al., Nature Biotechnology 2017).

Figure 9: Nature Biotechnology cover depicting one view of the central sinus of mouse bone marrow from the voluminous open imaging data published in this issue. *Reprinted by permission from Springer Nature, Nature Biotechnology, Three-dimensional map of non-hematopoietic bone and bone-marrow cells and molecules, Daniel L Coutu, Konstantinos D Kokkaliaris, Leo Kunz, Timm Schroeder, Copyright 2017.*

We are now using this approach to better quantify the location e.g. of different hematopoietic and mesenchymal and skeletal stem and progenitor cell populations, their hierarchy, and their possible interactions with their microenvironments in the bone marrow and other tissues.

Most recently, by incorporating antibody-based proximity ligation into the approach, we also succeeded to improve its sensitivity to the single-molecule level (Kunz et al., unpublished). This now allows the precise quantification of the location and concentration of many relevant molecular regulators and their interaction with other molecules, in space and simultaneously in relation to multiple cell types of interest. It will be exciting to see the individual molecular players in their specific locations and in relation to their producing and target cells, as opposed to the diffuse idea of an average even distribution throughout a tissue.

Of course, while this approach yields important quantitative single-cell data, it uses fixed tissue, thus not allowing the quantification of dynamics in live cells. Developing 3D live cell imaging approaches with the required depth, throughput, and importantly the duration to observe cellular processes for longer than a few hours remains an important technological problem for the community to solve in the future.

Some biological questions solved by long-term single cell quantification

Developing these approaches sent me on a 1.5 decade long detour and has been an important part of my group's work. However, technology development was always motivated and guided by the need of the biological questions to be solved. Here, I discuss a few of those long-standing questions in the hematopoietic system solved by long-term single cell imaging and quantification.

Finding the missing link: Hemogenic endothelium caught in the act

What is the origin of the first blood cells during development, and does hemogenic endothelium exist? This question remained controversial for more than a century.

Since the 1800s, it had been observed that the first blood cells in vertebrate embryos appear next to endothelial cells in all sites of de novo hematopoiesis – in the blood islands of the extraembryonic yolk sac, in the aorta of the aorta-gonad-mesonephros region within the developing embryo, and in the placenta. This led to several competing hypotheses about the specific embryonic cell type differentiating into the first hematopoietic cells. One possible explanation was a common precursor of endothelial and hematopoietic cells, the hemangioblast, which would simultaneously give rise to both cell types (Fig. 10) hence explaining their neighborhood (Hoppe et al., Nature Cell Biology 2014). Alternatively, the first blood cells could be generated from cell types close to, but different from, endothelium, e.g. in the embryonic subaortic mesenchymal patches and then transmigrate the endothelium into blood vessels (Hoppe et al., Nature Cell Biology 2014). The same could be true for cellular sources somewhere in the embryo far away from the first sites of appear-

ance of detectable blood cell numbers, with subsequent migration of the early blood cells to these sites either by the circulation within blood vessels, or by active migration outside the vessels (Tanaka et al., Cell Reports 2014). Finally, another explanation was the existence of hemogenic endothelium. In this case, endothelial cells would first be generated, and a subset would later differentiate into blood (Fig. 10). It would explain why nascent blood cells are found next to endothelium, and why endothelial and early hematopoietic cells share many molecular markers. However, this explanation could also hold true for all other above-mentioned hypotheses.

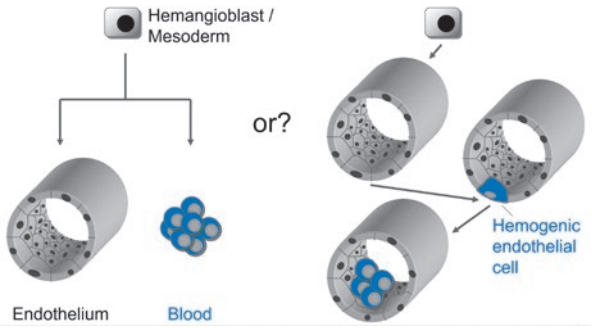

Figure 10: Possible cellular sources for the first blood cells during embryo-genesis. *Possible relationships between endothelium and blood. Left: endothelium and blood are independently created from one progenitor (hemangioblast). Right: blood is generated from specialized hemogenic endothelial cells. The existence of hemogenic endothelium could be proven by continuous long-term single-cell imaging of murine endothelial to hematopoietic transition in mesodermal cells derived from embryonic stem cells or primary embryonic mesoderm (Eilken et al., Nature 2009).*

Why was it so difficult to prove the existence of hemogenic endothelium? Since this process happens within the embryo, in mammals also deep in the uterus, it could never be observed live and at the single-cell level. The available snap-shot data from e.g. fixed and sectioned embryos could not exclude the other hypotheses discussed above as the sole and sufficient explanation. The existence of hemogenic endothelium thus remained disputed until 2009.

We therefore set out to establish a culture system for the relevant developmental processes, which would be optically accessible to be observed by time-lapse imaging. By using a 2-dimensional stromal co-culture system allowing the generation of blood and endothelium (as well as perivascular cells and cardiomyocytes) from mouse mesodermal cells, we could observe their differentiation at the single-cell level for many days. We used mesodermal cells derived either from embryonic stem cells differentiated into mesoderm in vitro, or directly from embryos at day 7.5 post fertilization. The use of embryonic stem cell derived mesoderm enabled the easier generation and use of fluorescent molecular reporter lines for the identification of specific endothelial and blood developmental and functional stages. The use mesoderm from the embryo on the other hand then allowed confirmation of observations with primary material directly from the embryo. By long-term imaging and tracking all progeny of individual mesodermal cells throughout their hemogenic differentiation, we were able to show that they indeed first go through endothelial stages – defined by morphology, molecular and functional markers – before further differentiating into blood cells. This provided prove for the long-disputed existence of hemogenic endothelium (Eilken et al., Nature 2009).

The provided evidence together with 3 simultaneously published studies with supporting evidence from alternative approaches indeed satisfied the field to accept the existence of hemogenic endothelium. It not only solved a long-standing dispute in developmental biology and provided some insight into the timing and control of a curios differentiation event at the birth of the hematopoietic system. It also defined a critical step in the generation of immature hematopoietic stem and progenitor cells with great potential for clinical therapy. Knowing that hemogenic endothelium exists guides the development of culture systems for the stepwise generation of desired cell types eventually leading to blood cell generation. It also guides the identification of the relevant molecular machineries and their manipulation for the induction of the hemogenic program in endothelial or other cells, e.g. through direct reprogramming. Indeed, the field saw a surge of activity leading to the confirmation of our findings in different vertebrates, improved understanding of endothelial to hemogenic transition and its molecular control (Swiers et al., Nature Communications 2013), and transfer of this knowledge to the continuously im-

proving attempts of generating definitive blood stem and progenitor cells from pluripotent and endothelial cells in vitro.

Lineage choice: Controlled by cell-intrinsic stochastic switches or instructed by cell-extrinsic signals?

How are lineage choice decisions made in differentiating multipotent progenitor cells? Are they made cell-autonomously by cell-intrinsic mechanisms or instructed by cell-extrinsic signals? This central and seemingly trivial question in hematopoietic stem and progenitor cell biology is discussed since the 1950s, and two major schools of thought with opposing basic concepts remain under discussion until today. The more obvious hypothesis assumes that lineage choice is instructed by signals from the microenvironment, which activate signaling pathways controlling the molecular programs inducing lineage choice, commitment and maturation (see also next chapter). However, colony assays in vivo and in vitro yield very heterogeneous lineage outputs with lineage choice frequencies which are constant only at the population level, but different and unpredictable between individual cells. This observation is hard to reconcile with the idea that lineage choice is under strict control of extracellular signals since all cells in the same culture medium should then behave the same. An alternative model of lineage choice therefore assumes cell intrinsic mechanisms which lead to different lineage choices with specific probabilities, respectively. In this case, the lineage choice of an individual cell is independent of its environment and cannot be predicted. However, at the population level, frequencies of a specific lineage are fixed. This is a very attractive model, since it would allow multipotent cells the required flexibility to differentiate into different cell types, while also being robust against dysregulated signals from the environment which would lead to overshooting uni-lineage differentiation (and thus the lack of required other lineages). In this model, the required adaptation of lineage output of the blood system depending on the body's need, in case of e.g. infections or lower oxygen environments, would be ensured by allowing survival and proliferation only of the required cell types after their lineage commitment, but not by influencing the lineage choice itself (see also selective model in the next chapter).

How would such a cell-intrinsic mechanism work to allow different outputs with specific frequencies? The most prominent hypothesis assumes lineage choice to be made by transcription factor networks, which are wired by protein-protein or protein-DNA interactions between its members. These circuits would lead to stochastic output, i.e. stable molecular end states with different defined probabilities, respectively, which would then drive different lineage choices. The stochasticity would be driven by random noise, e.g. from transcriptional bursts, and channeled into fixed probabilities for different outcomes by the wiring of the network with different interactions, feedback, feed-forward and dampening motives between different transcription factors and their genes. Indeed, molecular interactions between transcription factors involved in controlling hematopoietic lineage choice exist, making this an attractive and plausible model. However, none of these networks, their dynamics and their actual involvement in hematopoietic lineage choice could ever be quantified at the single cell level and linked to actual future cell fate choices – leaving the possibility that this is not more than a nice idea, and the use of the term *"stochastic"* here is just a euphemism for *"we have no clue what is actually going on"*.

The PU.1/GATA1 stochastic toggle switch does not initiate hematopoietic lineage choice

We therefore set out to quantify the actual dynamics of one paradigmatic central molecular switch of the hematopoietic transcription factor network, which was assumed to be responsible for lineage choice (Fig. 11).

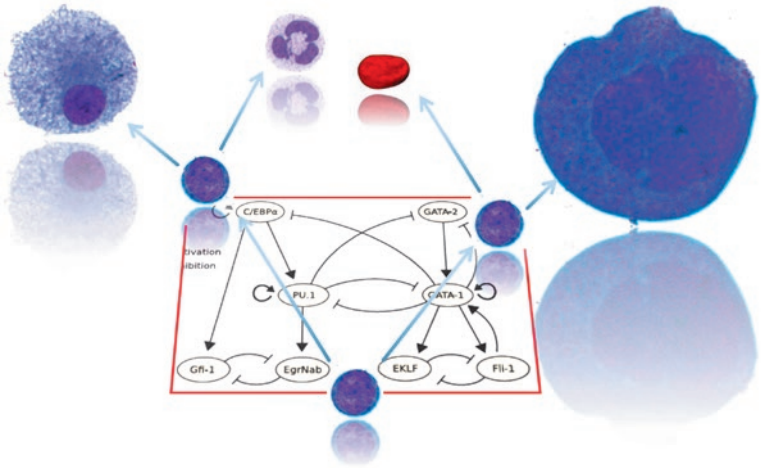

Figure 11: The putative transcription factor network underlying cell intrinsic stochastic lineage decision making during myeloid differentiation of hematopoietic stem cells. *Parts adapted from (Krumsiek et al., PloS One 2011).*

We non-invasively quantified the protein numbers of two transcription factors, PU.1 and GATA1, in living cells throughout their differentiation. These two proteins have long been described as lineage specific transcription factors for the monocytic/granulocytic and megakaryocytic/erythroid lineages of the hematopoietic system, respectively. Their overexpression can reprogram cells from one to the other lineage, respectively. Both protein can bind each other to inhibit the other's activity, and can auto-activate the transcription of their own genes, respectively. This wiring constitutes a toggle switch, where higher expression of one would lead to ever stronger expression and dampening of the stronger and weaker factor, respectively. A cell initially expressing both factors (e.g. before lineage choice), would thus flip into a state where only one of the factors would be expressed, leading to the lineage decision driven by this factor.

After years of technological optimizations, generating the required reporter mouse lines and manually tracking, we finally were able to simultaneously quantify the dynamics of protein expression for both transcription factors in living differentiating hematopoietic stem cells and all their

progeny over a week and up to 12 cell generations. By quantifying morphologies and molecular marker expression, we were able to detect lineage choice of the generated cells, and compare it to the previous molecular dynamics of the quantified factors.

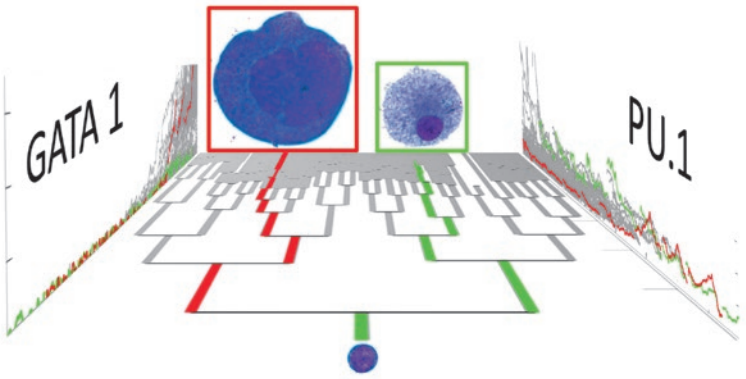

Figure 12: Non-invasive live single-cell quantification of intracellular protein numbers *of the lineage specific transcription factors PU.1 and GATA1 throughout the differentiation of hematopoietic stem cells into megakaryocytes, erythrocytes, monocytes or neutrophil granulocytes over up to 7 days and 12 generations.*

The average expression data found for both proteins at the end of the experiments was identical to what had been described in the literature. However, surprisingly, the expression dynamics we found before and during the time where lineage decisions were made were not compatible with what had been assumed in the field for decades (Hoppe et al., Nature 2016; Strasser et al., Nature Communications 2018). We could therefore show that the presumed stochastic PU.1/GATA1 switch is not used to initiate the monocytic/granulocytic versus megakaryocytic/erythroid lineage choice of differentiating hematopoietic stem cells. However, it would be premature to generalize this finding to conclude that stochastic molecular network switches are never responsible for cell-intrinsic cell fate control. It will be interesting to analyze whether this switch might be used to control the differentiation of other cell types, or if the same wiring, but of other molecules may be relevant for these cell fate choices.

Nature versus nurture: Lineage selection or instruction by hematopoietic cytokines?

Can cell-extrinsic cytokine signals influence the lineage choice of multipotent hematopoietic progenitors? Related to the question discussed above, this central and obvious question in hematopoietic stem and progenitor cell biology was intensively discussed since the 1950s. It surprisingly remained without a definitive answer for more than half a century – and while billions of US dollars' worth of cytokines were used annually for clinical therapy.

It was well-known that the lineage composition of hematopoietic colonies is influenced by the specific microenvironment these colonies developed in in vivo, or by the presence of hematopoietic cytokines in defined culture conditions in vitro. The types of living cells ultimately produced from multipotent blood progenitor cells can therefore be influenced by cell-extrinsic signals. However, this could be explained by very different fundamental mechanisms – lineage instruction versus lineage selection – which would both lead to the same final experimental observations described above. One possibility is that cytokine signaling directly influences the genetic and epigenetic programs controlling lineage choice – lineage instruction. However, it could also be possible that cells make their lineage choice independently of signaling pathway activity (see previous chapter), and cytokine signaling would only influence the survival and/or proliferation of already lineage committed cells. In this case, signaling pathways activated by cytokine signaling would only influence the molecular programs involved in cell survival or proliferation control, but have no influence on molecular lineage choice control.

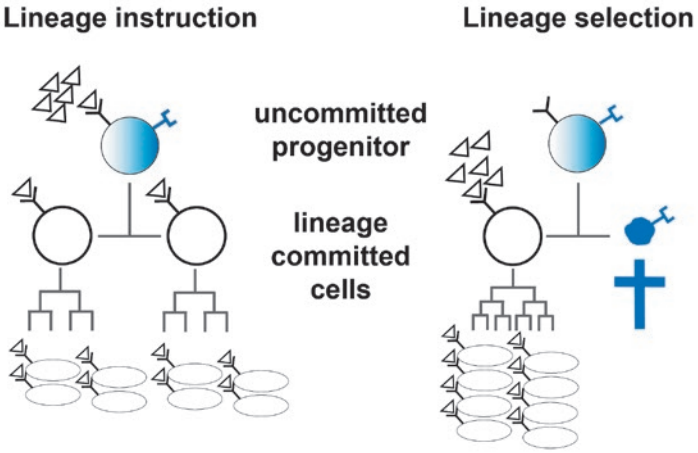

Figure 13: Alternative models – lineage instruction versus lineage selection – explaining the influence of cytokine signaling on colony lineage output. *The existence of lineage instruction on hematopoietic progenitor cells could be proven by long-term single cell imaging and tracking (Rieger et al., Science 2009). Adapted from (Rieger and Schroeder, Cell Cycle 2009).*

While conceptually very different and based on completely distinct molecular mechanisms and effects, it proved difficult to experimentally demonstrate the existence of lineage instruction. This was due to the difficulty to exclude the possibility of lineage choice control exclusively by selection with snapshot data. Cytokines undoubtedly support hematopoietic progenitor cell survival and proliferation. They can therefore clearly contribute to the enrichment of cells of a specific lineage by selection, and thus mask a potential additional contribution by lineage instruction. To prove the existence also of lineage instruction, it was therefore necessary to demonstrate the absence of cell death during the production of only one from multipotent progenitors depending on cytokine treatment. The problem here is the days-long delay between lineage decision making and the subsequent maturation leading to the cellular phenotypes required to detect their commitment to their lineage. During this time, hematopoietic progenitor cells proliferate quickly, producing dozens to hundreds of differentiated progenies. Excluding the possible death of one

individual cell during that time – which could have cell-intrinsically committed to another lineage and was then killed due to the lack of its required lineage specific cytokine – was impossible with previous technologies.

We therefore used continuous long-term observation of all individual cells produced from individual granulocyte-monocyte progenitor (GMP) cells over many days until their lineage commitment could be reliably detected. GMPs were cultured under chemically defined conditions with the presence of either cytokine G-CSF or cytokine M-CSF, leading to the production of only granulocytic or monocytic cells at the end of the cultures from the same starting GMP population, respectively. This allowed us to quantify the frequency of cell death and division events for all of the progeny of the initial cells. Between these culture conditions, we could not find relevant differences in cell proliferation, or in cell death. Importantly, the frequency of cell death events was not sufficient to explain the lack of production of granulocytic cells under M-CSF conditions or monocytic cells under G-CSF conditions. Thus, it was not the selective killing of the "other lineage" cells under lineage specific cytokine culture conditions leading to a uni-lineage differentiation output. Lineage choice must therefore have been directly instructed by signaling activity.

Signaling pathways activated by cytokine receptors therefore must change the molecular programs controlling lineage choice. This insight not only clarifies a long-standing dispute about a core mechanism of multipotent progenitor cell fate control. It is important also because it offers an excellent experimental system to now identify the pathways and relevant molecular mechanisms underlying lineage choice.

This should be easy. One would think that a simple comparison of the intracellular signaling pathways activated by the opposing cytokine receptors would identify the pathway(s) responsible for one or the other lineage choice. However, it turns out to be surprisingly difficult. Despite their opposing effects on lineage choice, the receptors for G-CSF and M-CSF both activate many signaling pathways, and most of them overlapping and highly interconnected. Add the shared confounding effects of both cytokines on cell survival, proliferation, maturation, adhesion and activation, it becomes very demanding to disentangle the effect of these in-

dividual pathways on the different cell fates, and to identify those influencing the molecular control of lineage choice. We used a molecular loss of function approach combined with long-term single-cell quantification of GMP lineage choice to identify the relevant pathway (combination) mediating the monocytic lineage instructive effect of M-CSF. M-CSF receptor deficient GMPs were rescued with mutants of the receptor which activate only one or a subset of the many pathways activated from the eight intracellular Tyrosine residues of the M-CSF receptor. We could finally show that src family kinases are sufficient to instruct monocytic lineage choice. However, they were also not strictly required since the other signaling pathways activated by the M-CSF receptor could apparently compensate for their absence (Endele et al., Blood 2017). Overall, it remains obscure how signaling from activated receptors exert their specific effects on cell fate choices.

The above experiments were based on the assumption that different combinations of intracellular signaling pathways activated by cytokine receptors are responsible for their specific effects. However, there is another possibility to encode specificity – dynamics of signaling pathway activity. The idea would be that different dynamics of pathway activity can activate different molecular target programs (Fig. 14). This would allow different cytokine receptors which activate the same intracellular pathway(s) to still have specific effects.

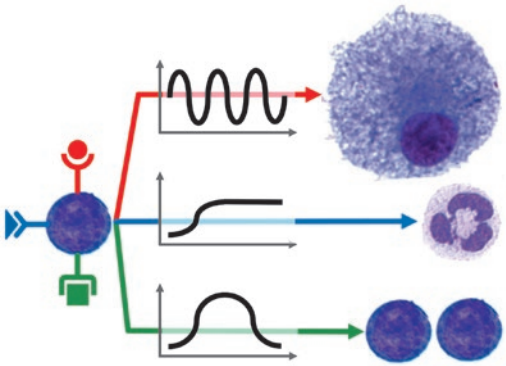

Figure 14: Different activity dynamics of the same pathway induced by different cytokine receptors could explain cytokine-specific effects on cell fate choices.

There is beautiful precedence for this concept from cell lines. However, due to the technological demands, the concept has never been tested for primary hematopoietic stem and progenitor cells. It, again, requires the demanding combination of non-invasive high-frequency quantification of signaling pathway activity over hours with the long-term quantification of future cell fate choices over days – in living single cells and all of their progeny. We have therefore developed approaches for the high-frequency live quantification of transgenic biosensors for signaling pathway activity – simultaneously for many different pathways in primary mouse and human stem and progenitor cells. Indeed, we find highly heterogeneous signaling pathway dynamics in individual cells of purified progenitor populations, despite stimulation with the same cytokine. It will now be interesting to link these specific dynamics to the future cell fate choices of individual cells to add another layer of molecular fate control to our understanding of hematopoietic cell fates.

Asymmetric cell division of hematopoietic stem and progenitor cells

Are hematopoietic stem and progenitor cell fates controlled by asymmetric cell division? This question has been under dispute for many years. In asymmetric cell division, the future asymmetric fates of two sister cells are fixed during the division of their mother cell. This could be due to e.g. the asymmetric inheritance of intracellular cell fate determinants into the two daughters, or the orientation of the division plane leading to unequal niche access of the two sisters after division (Fig. 15). It would be an attractive explanation how the number of stem cells could be kept constant throughout the body without the need of complex and potentially vulnerable systemic feedback mechanisms. Each stem cell would, under homeostatic conditions, give rise to one daughter which would go on to differentiate and produce the different cell types of the blood system, and one daughter which replaces its mother as a stem cell, thus keeping the stem cell pool size constant. While it has beautifully been shown to exists in other cell types, and some textbooks include asymmetric division even in the definition of hematopoietic stem cells, many researchers do not believe it exists in these cells. Again, the reason for this long-standing dispute is the lack of adequate technology. Observation of either asymmetric fates or asymmetric inheritance of intracellular molecules or niche

access alone is not sufficient to prove the existence of asymmetric cell division. To prove the existence of this peculiar, beautiful and therapeutically attractive mechanism, asymmetric events during division and asymmetric future daughter cell fates have to be quantitatively detected, and shown to correlate in the same cells (Schroeder, Cell Stem Cell 2007). Again, this requires continuous live molecular single-cell imaging in combination with long-term single-cell fate quantification of rare and difficult to purify and culture hematopoietic stem cells.

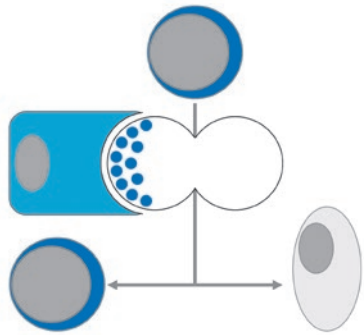

Figure 15: Asymmetric cell division.

Asymmetric fates of hematopoietic stem cell daughters had long been described. After the first years of imaging the potential asymmetric inheritance of intracellular molecules and organelles, we had also found some which are asymmetrically segregating during divisions. However, it took us almost a decade to be able to link those two together. For years, different combinations of asymmetries in inheritance during mitosis and in future fates did not correlate with each other, thus not allowing the conclusion that asymmetric molecular inheritance has any functional relevance. Recently, however, we were finally able to find clear correlations between the inheritance of specific molecules and organelles during hematopoietic stem cell divisions, and their future metabolic activation and differentiation (Loeffler et al., in revision). This only happens in a low but reproducible percentage of divisions, and the differences in inheritance between sisters are usually less than two-fold, thus requiring precise quan-

titation. However, the reproducible frequency, and the clear correlations found now finally allow us to conclude that asymmetric cell division exists in hematopoietic stem cells. It is an orthogonal and high-level regulatory mechanism controlling hematopoietic stem cell fates with a lot of potential for novel insights. It will now be exciting to unravel the molecular mechanisms, target effector programs and possibilities for molecular manipulation of this process for therapeutic intervention.

Where to next?

After 1.5 decades of my own independent research group, we have established important and unique technologies for long-term single-cell quantifications. These approaches work, both in my own, and in other groups. But they still need expert knowledge and further optimizations. They have contributed to answering diverse long-standing questions in different cell types and molecular systems. After slow and sometimes tedious development of technology, we are now getting faster and faster in successfully applying it to novel biological questions. In addition to quantitative observations, the precise molecular manipulation, both through fine control of fluidics, and increasingly through fast optogenetic approaches, will become important for unravelling the functional role of specific molecules in regulatory networks. With increasing numbers of well-defined culture systems for different cell and tissue types, more and more biological questions become available for long-term single-cell imaging and quantification. The advent of organoid cultures of many solid tissues in combination with light-sheet imaging will lead to a surge of imaging data to be analyzed for the same concepts, but will also require novel custom software components. Long-term in vivo single-cell imaging with sufficient throughput and duration remains a crucial goal in the field, but will likely require novel imaging modalities for many of the biological questions at hand. Improved automation, algorithms and software remain a crucial requirement, and a lot of work still has to be done in this area. After mostly working on murine systems for the first many years due to their better experimental accessibility, reproducibility and the availability of transgenic reporter systems, we have begun to increasingly build on the gathered experience for the analysis also of human cells. Finally, I am convinced that quantification of cellular and molecu-

lar dynamics will enable the next level of insights in high-throughput screening approaches in e.g. for new drugs in the pharmaceutical industries, and importantly also in clinical diagnosis, patient stratification, and the development of novel therapies.

I am very much looking forward to contributing to these and other areas of research. After many years of establishing required technologies, I feel that we can now finally tackle many biological and medical questions much more efficiently. We are ready to really get started. I will likely feel the same again in another 1.5 decades.

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