Pioniergeist
From past innovations to future developments:
Miltenyi Biotec 1989 – 2019 and beyond

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The cover image shows a snake embryo after tissue clearing and staining for β-III tubulin (axonal marker, cyan) and FoxP2 (neuronal marker, magenta). The image was generated by light sheet microscopy (see article on p. 10).

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Dear Researcher,

In 1989, Stefan Miltenyi started the company based upon a technique to isolate cells gently and effectively – MACS® Technology. This elegant method quickly became the prime method of cell separation. Within the next three decades, Miltenyi Biotec grew into an organization of about 2,500 employees, offering a portfolio of more than 17,000 products. Continuous development of cutting-edge solutions has driven the company to the forefront of biomedical research and cell and gene therapy.

One of Miltenyi Biotec’s most recent innovations provides an unheard-of insight into the physiology and pathophysiology of a tissue or cell sample – the MACSima™ Imaging Platform (see article on p. 16), which was introduced earlier this year. Further strengthening Miltenyi Biotec’s expertise in imaging and microscopy, the LaVision BioTec team with their vast expertise in light sheet microscopy and 3D imaging joined the company in 2018. These technologies perfectly complement Miltenyi Biotec’s broad portfolio of cell analysis solutions.

Over the years, Miltenyi Biotec’s persistent efforts in the field of cell and gene therapy led to numerous groundbreaking innovations, such as the FDA-approved CliniMACS® CD34 Reagent System. In their constant quest for novel cancer therapies, the company initiated four clinical trials based on chimeric antigen receptor (CAR)–expressing T cells in 2018. CAR T cells are among the most promising approaches towards the development of new immunotherapies and, at Miltenyi Biotec, we are excited to contribute actively to this intriguing field. Current developments include a technique that enables the tightly controlled activation of CAR T cells, which will further enhance the versatility and potential of CAR T cells (p. 40).

The Miltenyi Biotec team always endeavored to develop advanced solutions for biomedical research in collaboration with researchers and clinicians across the globe. Many of these collaborative projects evolved into pioneering concepts enabling the scientific community to enter new avenues.

In this anniversary issue of MACS&more, distinguished scientists share their research results and scientific perspectives. Robin Vigouroux and Alain Chédotal provide a deep insight into the different methods of tissue clearing and the resulting options for 3D imaging by light sheet microscopy (p. 10).

Graft engineering for hematopoietic stem cell transplantation has been refined continuously over the course of many years. Michael Maschan gives an overview of the different approaches and shares his experience from clinical studies using CD19− B cell– and TCRαβ− T cell–depleted grafts (p. 21).

Tumor antigen–presenting dendritic cells (DCs) have a huge potential to initiate antitumor immune responses. Jolanda de Vries, Kalijn Bol, and Gerty Schreibelt highlight the use of naturally occurring blood-derived DC subsets as vaccines in clinical trials (p. 37).

Scott Tasker and Graham Lord are going to start a clinical trial towards a cellular therapy of Crohn’s disease in mid 2019. Their recent research results suggest that a lack of gut-homing regulatory T cells causes the symptoms of the disease. In their trial they aim to compensate for this deficiency and ultimately prevent the inflammatory imbalance by administering purified gut-homing regulatory T cells (p. 44).

Hyun-Dong Chang and Andreas Radbruch take a look back at the beginnings of MACS Technology and present one of their most recent projects using this technology for the enrichment of rare bacteria in the context of microbiota analysis (p. 46).

We hope you enjoy reading this special issue of MACS&more and catch the excitement of Pioniergeist.

Best wishes,
MACS&more team
At the forefront of innovation – research and development at Miltenyi Biotec 1989–2019 and beyond

In 1989 Miltenyi Biotec started its business with a single product for cell isolation based on MACS® Technology. The company quickly developed into a provider of comprehensive solutions for biomedical research. In close collaboration with researchers and clinicians worldwide, Miltenyi Biotec continuously brings forth cutting-edge technologies suitable for basic research and translation into clinical settings. Here we discuss some examples of the powerful innovations that Miltenyi Biotec has made available to the scientific community.

MACS® Technology – the solid foundation for cell research and cellular therapy

A sophisticated cell separation technology based on superparamagnetic MicroBeads coupled to antibodies, a permanent magnet, and columns was the starting point for Miltenyi Biotec in 1989. With its gentle mechanism and the availability of hundreds of cell separation reagents, MACS® Technology became the prime method for the isolation of viable, functional cells in biomedical research. Right from the start, Miltenyi Biotec’s Research & Development Department also had the finger on the pulse of clinically relevant topics. This resulted in the development of the “CD34 Progenitor Cell Isolation Kit” in 1993. Based on MACS Technology, this kit enabled the effective enrichment of CD34+ hematopoietic stem cells (HSCs) from blood products. When it was released to the market, it was only a matter of time before this tool caught the attention of one of the big players in the biotech industry.

Back in those days, Amgen, the largest independent biotech company worldwide today, was working on a project aiming to fight breast cancer with high-dose chemotherapy. To Amgen, MACS Technology was the method of choice to enrich autologous CD34+ HSCs for immune reconstitution and at the same time remove potential cancer cells from the graft that might otherwise cause a relapse. Consequently, in 1994, Miltenyi Biotec and Amgen initiated a joint venture, called AmCell, which led to the development of a sophisticated instrument for automated, clinical-scale cell enrichment. However, over the years it turned out that the approach pursued by Amgen did not bring forth the desired results, which subsequently led to the abandonment of the joint venture in 2002. What remained is the powerful CliniMACS® System, which continues to be the basis for numerous endeavors.

In December 1997, the CliniMACS CD34 System components received the CE mark, registered as medical devices in Europe, and were officially introduced into the market. Over the intervening years, tens of thousands of patients were treated with CD34+ cells enriched by the CliniMACS System in clinical studies conducted by numerous scientists and clinicians. Finally, in 2014, Miltenyi Biotec’s constant quest for advancing cellular therapy came to fruition: The US Food and Drug Association (FDA) approved the CliniMACS CD34 Reagent System – a method to deplete T cells as an effective means of graft-versus-host disease (GVHD) prophylaxis in patients with acute myeloid leukemia undergoing allogeneic stem cell transplantation in first complete remission.
The entire process occurs in the closed system of a single tubing set – from cell separation, activation, transduction, and expansion through to final formulation.

Cell manufacturing on the CliniMACS Prodigy is thus largely independent of an expensive dedicated, centralized GMP facility; instead the cells can potentially be processed anywhere. Therefore, the CliniMACS Prodigy can serve two entirely different concepts of cell manufacture: i) centralized manufacturing, meaning that the starting material is transported to one principal facility where the cells are processed, and the final cell product is then delivered to the point of care; or ii) decentralized manufacturing where cells are processed at multiple sites close to the point of care, i.e., close to the patient.

**Solutions for automated flow cytometry – MACSQuant® Analyzers**

In 2008, at a time when flow cytometers were humongous machines, Miltenyi Biotec began to launch a series of benchtop flow cytometers with extremely small footprints, the MACSQuant® Analyzers. The first instrument was equipped with three lasers providing nine optical parameters. Over the years, the MACSQuant Flow Cytometers were continuously refined and remarkable features were added. Today, four different flow cytometers are available with up to 16 detection channels. The instruments provide a high level of automation, including instrument calibration, compensation, as well as automated startup, cleaning, and shutdown. Moreover, up to 384 samples can be processed fully automatically in one go, from sample labeling and uptake through to data acquisition and analysis. All these features were developed with the user in mind. MACSQuant Flow Cytometers are designed to make sophisticated flow cytometry analysis available to lab personnel regardless of expertise.

MACSQuant Analyzers enable the reliable analysis even of rare cells, such as antigen-specific T cells. An integrated MACSQuant Column offers the option to pre-enrich the target cells based on MACS® Technology, thus eliminating bulk cells from the sample. As a result, the frequency of the cells of interest increases, which enables further analysis.

**Figure 2: Enrichment of Aspergillus-specific CD4+ T cells enables reliable flow cytometry analysis.**

Human PBMCs were left untreated or were stimulated with *Aspergillus* lysate for 6 hours, which induced expression of the activation marker CD154. However, the proportion of CD154-expressing CD4+ T cells was very low (0.35%), which impeded further characterization. Enrichment of the activated CD4+CD154+ T cells by the integrated MACSQuant Column on the MACSQuant Analyzer 10 resulted in a substantially higher percentage of these cells, thus facilitating further analysis.

Continuous efforts of Miltenyi Biotec’s Research & Development Department in cooperation with international researchers led to the development of a multitude of applications in the fields of graft engineering, immunotherapy, and tissue regeneration using the CliniMACS System (see info box).

The versatility of the CliniMACS System resulted in more than 200 Investigational New Drug (IND) applications or Device Exemptions (IDEs) at the US FDA and over 150 clinical studies in the EU and rest of the world.

Another huge step forward in the cell therapy field was made with the introduction of the CliniMACS Prodigy® in 2013. The device is capable of performing complex cell manufacturing workflows, such as the generation of CAR T cells, within a single process setup. Current methods for the manufacture of engineered T cells encompass various complex and labor-intensive procedures. In contrast, the CliniMACS Prodigy T Cell Transduction Process enables the generation of CAR T cells in a standardized, fully automated way.

**CliniMACS® System offers versatile options**

The CliniMACS System (fig. 1) provides the basis to explore a multitude of cell therapeutic approaches in the fight against serious diseases, including cancer:

- Enrichment of CD34+ cells and depletion of T cell subsets and B cells from HSC grafts
- Enrichment of T cell subsets and NK cells for donor lymphocyte infusion
- Generation of dendritic cell (DC)-based vaccines using enriched blood DCs or monocyte-derived DCs
- Manufacture of chimeric antigen receptor (CAR) T cells and other genetically modified cells
- Expansion and differentiation of adherent mesenchymal stem cells (MSCs) and pluripotent stem cells
Revolutionary microchip-based technology for sterile and safe fluorescence-based cell sorting – the MACSQuant® Tyto® Cell Sorter

Magnetic cell separation by MACS® Technology is the perfect choice in many settings. However, certain cell populations that do not express a unique marker for specific labeling are difficult to isolate based solely on magnetic cell separation. In 2018, Miltenyi Biotec released the MACSQuant® Tyto® Cell Sorter, a benchtop instrument that allows sophisticated fluorescence-based sorting strategies using multiple markers. Based on microchip technology, the MACSQuant Tyto enables high-speed cell sorting in a closed cartridge system. Unlike on droplet sorters, cells do not get decompressed, and no charge is applied, resulting in high viability rates and maintained functionality. Importantly, the closed, sterile cartridge system protects the operator from potentially infectious materials and the cell product from contaminations. Fully automated processes eliminate the requirement of extensive specialized technical expertise, which make the MACSQuant Tyto amenable to all lab professionals, not only flow sorting experts.

Naive CD4+CD25+CD127lowCD45RA+ regulatory T (Treg) cells, for example, which are a promising cell type for the development of therapies in the context of transplantation and autoimmunity3, can be isolated using the combination of MACS Technology for pre-enrichment of CD25+ cells and the MACSQuant Tyto for further sorting based on CD4, CD127, and CD45RA (fig. 3).

Label-free cells after flow cytometry-based cell sorting and magnetic cell separation

For many applications, it is desirable to obtain label-free cells after cell isolation. To that end, Miltenyi Biotec developed the REAlease® Technology, which is designed for the removal of all labels from cells after flow cytometry-based cell sorting or magnetic cell separation. REAlease Technology relies on recombinantly engineered antibody fragments instead of complete antibodies to label specific cell surface markers. The single antibody fragments have a low affinity for cell surface epitopes. However, when the fragments are multimerized as a complex, they bind epitopes on target cells with high avidity and enable effective labeling, comparable to conventional antibodies. After cell isolation, the complex can be disrupted by adding a single reagent, which ultimately results in monomerization of the antibody fragments and consequently in the removal of labels.

Using REAlease Fluorochrome Technology, fluorescent labels can be completely released after cell sorting, which provides numerous benefits: I) Marker epitopes become available again for downstream applications that require free epitopes, such as cell culture experiments and cell analysis (fig. 4). II) Fluorescence channels that were blocked by the fluorochromes used for sorting become available again for relabeling in microscopy or flow cytometry experiments, for example. III) Sequential flow sorting cycles for the isolation of specific cell subsets are made easier.

REAlease MicroBead Technology is the complementary technique for magnetic cell separation with superparamagnetic MicroBeads. After removal of the label from the isolated cells, the cells can be relabeled to obtain a certain cell subset, for example.

REAlease Fluorochrome Technology was awarded with the technology prize at the Pharmaceutical Flow Cytometry & Imaging 2018 conference in Stevenage, UK. Delegates of the European Laboratory Research & Innovation Group (ELRIG) nominated this technology, underlining its importance for biotechnology and pharmaceutical research.
**Figure 4: Isolation of Treg cells using the MACSQuant® Tyto® Cell Sorter and REAlease® Technology.** Expression levels of antigens used for identification of Treg cells are low. Therefore, bright fluorescent antibody conjugates are required for effective discrimination and sorting of these cells. In this experiment, human PBMCs were labeled with an antibody panel optimized for flow sorting, composed of REAlease CD25-APC, human and conventional CD4-PE-Vio® 770, human and CD127-FITC, human antibodies (A). Upon isolation of the CD4+CD25+CD127+ Treg cells (B), the REAlease CD25-APC label was selectively removed from the target cells (C). This allowed for relabeling of the CD25 epitope with the CD25-PE conjugate and reuse of the APC detection channel to address the Treg cell–specific transcription factor FoxP3 with the Anti-FoxP3-Vio 667 antibody. Flow cytometry analysis to assess identity and purity of the CD25+ Treg cells in the sorted fraction could thus be performed at maximum sensitivity (D). Data courtesy of Petra Bacher, Charité – University Medicine Berlin, Berlin, Germany.

**REAfinity™ Recombinant Antibodies for greater reproducibility**

A few years ago, Andrew Bradbury, Andreas Plückthun, and 110 signatories raised an important discussion about the reproducibility of research results that rely on antibodies. They estimated that, in the USA alone, about 350 million US Dollars are wasted annually due to poorly defined antibodies. In their commentary the authors emphasized the need for recombinant antibodies to improve reproducibility and hence save money.3 Miltenyi Biotec introduced the portfolio of REAfinity™ Recombinant Antibodies in 2012. Over the years, the range of these antibodies was expanded continuously; today more than 1000 specificities are available for human and mouse. REAfinity Antibodies are derived from a defined set of genes, and the production process is highly standardized. Accordingly, these antibodies are highly consistent in composition from lot to lot, whereas traditional mouse monoclonal antibodies can contain
contaminating hybridoma-derived Ig light chains (fig. 5).

REAfinity™ Antibodies include a universal human IgG1 Fc region, which reduces the complexity of experiment planning because only a single isotype control is required during flow cytometry analysis. Moreover, the IgG1 Fc region is specifically mutated to abolish any binding to Fcγ receptors and eliminate the associated background signals. Miltenyi Biotec’s recombinant antibodies are subject to strict analytical, biochemical, and cell-based quality controls and are validated for flow cytometry.

Miltenyi Biotec is represented in the antibody validation group for flow cytometry initiated by the Global Biological Standards Institute. This working group aims to develop “guidelines and scoring to drive the use of high quality antibodies in research”.

**Gentle, yet effective tissue dissociation – gentleMACS™ Technology**

Many biomedically relevant research techniques, such as cell isolation by MACS™ Technology, flow cytometry analysis, cell sorting, or cell culture, require single-cell suspensions as starting material. Preparation of cell samples from blood products is relatively easy to accomplish. However, in order to obtain single cells from solid tissue, careful sample preparation is required, and manual methods are challenging in this respect. In 2009, Miltenyi Biotec launched the gentleMACS™ Technology which combines automated mechanical tissue dissociation with enzymatic digestion and enables gentle, yet effective generation of single-cell suspensions at the push of a button. Automated processes on a gentleMACS Instrument (fig. 6) using the patented gentleMACS C Tubes and numerous MACS Tissue Dissociation Kits make for a high level of standardization. More than 20 different kits were developed to provide optimized conditions for the dissociation of tissues from mouse or human. All components of the gentleMACS Technology were fine-tuned to yield the maximal amount of viable single cells while preserving cell functions and characteristics, including cell surface epitopes. Reliable sample preparation provides a solid basis for successful downstream cell-based experiments.

**High-content imaging with the MACSima™ Imaging Platform**

In recent years, Miltenyi Biotec has expanded its research and development efforts into two important, biomedically relevant technologies – imaging and microscopy. In spring 2019, Miltenyi Biotec introduced the MACSima™ Imaging Platform, which enables iterative cycles of immunofluorescent staining, imaging, and erasure of the fluorescence signal. Fully automated processes on the MACSima Imaging System (fig. 7) allow for the analysis of potentially hundreds of proteins or other antigens on a single sample, whereas current immunofluorescence

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**Figure 5:** Mass spectrometry analysis of purified REAfinity Antibodies and hybridoma-generated antibodies. (A) Two examples of hybridoma-generated monoclonal antibodies contain a second Ig light chain with a molecular weight of approximately 23,635 Da. In the bottom left example, the amount of the contaminating light chain exceeds by far the productive light chain of approximately 24,142 Da. (B) Two examples of recombinant REAfinity Antibodies show pure light chain populations.

**Figure 6:** gentleMACS Octo Dissociator with Heaters, gentleMACS Tubes, and Tissue Dissociation Kit.
AmCell. The pentaspan transmembrane Another important discovery, CD133 as a Scientific findings translated into biomedical research solutions

Research at Miltenyi Biotec resulted in major scientific discoveries, also in cooperation with scientists from across the world. One of the most significant contributions was the discovery of CD303 (BDCA-2), a specific marker for plasmacytoid dendritic cells (pDCs) that appears to have a dual function of antigen internalization for presentation to T cells and potent inhibition of IFN-α/β induction. Miltenyi Biotec’s CD303 (BDCA-2)-specific antibodies and cell separation reagents have since facilitated pDC characterization and isolation.

Another important discovery, CD133 as a marker for human hematopoietic stem and progenitor cells, was accomplished in the context of the joint venture with Amgen, i.e., AmCell. The pentaspan transmembrane glycoprotein (also known as prominin-1) has also been used as a marker for cancer stem cells in various tumor types. CD133+ cells were used in many studies related to autologous and allogeneic stem cell transplantation as well as regenerative medicine.

A smart technique for the detection and isolation of live, cytokine-secreting cells was developed in close collaboration with Andreas Radbruch’s group at the University of Cologne. This method provided the basis for the CliniMACS® Cytokine Capture System (IFN-gamma), which enables the clinical-scale enrichment of antigen-specific CD4+ and CD8+ T cells.

Miltenyi Biotec is actively involved in the scientific community, not only by continuously interacting with scientists worldwide, but also by sharing discoveries. Researchers from Miltenyi Biotec have published and contributed to hundreds of peer-reviewed articles over the years.

Looking back and ahead

Back in 1989, when Miltenyi Biotec started off with a single product, it was not conceivable that the company one day would make an FDA-approved system available for use in the treatment of patients with acute myeloid leukemia. The development of numerous unique solutions over time has enabled researchers and clinicians to break new ground, resulting in tens of thousands of publications in many research fields. Novel technologies such as the MACSQuant Tyto Cell Sorter or the MACSima Imaging System are just beginning to unfold.

Many clinical trials based on the CliniMACS System are currently in progress worldwide, in a quest to tackle serious diseases. What was a vision in the early days of Miltenyi Biotec has long become reality – providing the opportunity to develop innovative cellular therapies.

References

Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. MACS® GMP Products are for research use and ex vivo cell culture processing only, and are not intended for human in vivo applications. For regulatory status in the USA, please contact your local representative. The CliniMACS Reagents are manufactured and tested under a quality system certified to ISO 13485 and are in compliance with relevant GMP guidelines. They are designed following the recommendations of USP <1043> on ancillary materials. The CliniMACS® System components, including Reagents, Tubing Sets, Instruments, and PBS/EDITA Buffer, are designed, manufactured and tested under a quality system certified to ISO 13485. In the EU, the CliniMACS System components are available as CE-marked medical devices for their respective intended use, unless otherwise stated. The CliniMACS Reagents and Biotin Conjugates are intended for in vitro use only and are not designated for therapeutic use or direct infusion into patients. The CliniMACS Reagents in combination with the CliniMACS System are intended to separate human cells. Miltenyi Biotec as the manufacturer of the CliniMACS System does not give any recommendations regarding the use of separated cells for therapeutic purposes and does not make any claims regarding a clinical benefit. For the manufacturing and use of target cells in humans the national legislation and regulations – e.g. for the EU the Directive 2004/23/EC (“human tissues and cells”), or the Directive 2002/98/EC (“human blood and blood components”) – must be followed. Thus, any clinical application of the target cells is exclusively within the responsibility of the user of a CliniMACS System.

In the US, the CliniMACS CD34 Reagent System, including the CliniMACS Plus Instrument, CliniMACS CD34 Reagent, CliniMACS Tubing Sets TS and LS, and the CliniMACS PBS/EDITA Buffer, is FDA approved as a Humanitarian Use Device (HUD), authorized by U.S. Federal law for use in the treatment of patients with acute myeloid leukemia (AML) in first complete remission. The effectiveness of the device for this indication has not been demonstrated. All other products of the CliniMACS Product Line are available for use only under an approved Investigational New Drug (IND) application or Investigational Device Exemption (IDE). CliniMACS MicroBeads are for research use only and not for human therapeutic or diagnostic use. In the US, the CliniMACS Product T Cell Transduction Process is available for research use only.
Tissue clearing and light sheet microscopy – the perfect match for 3D imaging

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Recent developments for visualizing biological samples in three dimensions (3D) has led the biological community to re-interpret complex biological systems. This has been made possible due to converging advances in light sheet fluorescence microscopy (LSFM), tissue clearing, and molecular targeting. Here, we describe the recent advances, current limitations, and outlooks in the field.

Light sheet microscopy – a not so old principle

Since the discovery of the light sheet microscope (LSM) by Siedentopf and Zsigmondy in 1903, using illumination of colloidal solutions, LSM has become an incredibly attractive choice for biologists across the globe. Indeed, it was elected method of the year by Nature Methods in 2014. Since, many developments in LSM have been made, and we will attempt to summarize these advances.

Instead of illuminating the entire sample (resulting in photobleaching), a light sheet microscope shines an orthogonal sheet of light on the sample, eliminating the optical aberrations of the classical wide-field microscopes. The detection arm is placed perpendicular to the illumination laser. This simple and elegant principle provided for the first time the ability to image large biological samples without depth of scattering. Several groups applied this principle to view the surface of the Drosophila melanogaster compound eye or rhodamine-labeled pig cochlea. Strikingly, it was not until a century later that the LSM was used to its full potential. In 2004, the compatibility of LSM with fluorescence signals drew much attention. However, this remained restricted to optically clear samples such as zebrafish larvae. In 2007, Dodt and colleagues developed the ultramicroscope, which combined tissue clearing (BABB, see next chapter) and light sheet fluorescence microscopy (LSFM) to visualize GFP-positive neurons in large biological tissues such as the brain.

Although standard LSM provided an incredible step forward for biological sample imaging, several issues remained. Due to the Gaussian nature of the light sheet, the light distribution on the sample implies that only a specific area could reach maximum optical resolution (Rayleigh range). However, in LSM this range is notoriously short. Some commercial microscopes have addressed this by acquiring the area of highest resolution and tiling, a feature called dynamic focusing (LaVision Biotec, a Miltenyi Biotec company). However, this results in longer imaging times and larger datasets. Other strategies have been developed in parallel. Saghafi et al. proposed an ingenious setup by combining two to three aspheric lenses, which transformed the Gaussian beam into an elliptical beam, with a cylindrical lens. That way they could create an ultra-thin light sheet. Recent developments of non-Gaussian beams, such as Bessel beams or lattice light sheet, have helped reach nanometer resolution.

Multiview light sheet microscopy has been used as another strategy to reach nanometer axial and lateral resolution. In any imaging system, axial resolution remains the poorest. To circumvent this drawback, several groups have developed a method of imaging the sample in multiple orthogonal views and merging the datasets to enhance the low axial resolution by the much improved lateral resolution, resulting in an isotropic image. Recently, the Keller group has made great improvements to these microscopes by creating adaptive optics combined with multiview light sheet microscope, SiMView, to image the developing mouse embryo from gastrulation to early embryogenesis with single-cell resolution. McDole et al. were able to image the entire developing mouse embryo for up to 48 hours, a tissue whose size increases by 250-fold during this time period, without losing the tissue from the field of view.

Altogether, recent technological advances in light sheet microscopy have allowed us to gain a better understanding of developmental biology by providing i) rapid illumination – resulting in extremely low phototoxicity and photobleaching and ii) thin light sheets coupled to multiview scanning – resulting in high axial and lateral resolution.
### Tissue clearing – a 3D revolution

The notion of tissue clearing began over a century ago with the work of the German anatomist Werner Spalteholz. He achieved clearing of tissues by testing different organic solvents, such as combinations of benzyl alcohol and methyl salicylate\(^5\). Due to the heterogeneous refractive index (RI) in a given biological sample, light is both absorbed and diffracted, resulting in an opaque appearance. However, if one harmonizes the RI of a biological tissue, by removing water and lipids, then immersion of the tissue in a medium with the same RI would render it “transparent” (fig. 1). For a long time, this practice was restricted to medical diagnostics with preparations such as Murrays clear (benzyl alcohol and benzyl benzoate, BABB) being used by embryologists. It was not until the early 2000’s that the group of Hans Ulrich Dodt made this technique widely attractive to the scientific community. By combining ethanol dehydration and RI matching in BABB, Dodt et al. applied tissue clearing to light sheet fluorescence microscopy to visualize axons expressing a green fluorescent protein (GFP) in the whole brain of transgenic mice and to show blood vessels by making use of autofluorescent signals\(^6\). The sheer number of tissue clearing protocols can be reduced to two main approaches, organic solvents and aqueous solutions. We will not attempt to give a complete picture of tissue clearing protocols, but rather focus on recent advances in the most frequently used protocols. For a more thorough overview on protocols refer to these reviews\(^1\)–\(^1\)\(^9\).

Organic solvents have remained an attractive choice for laboratories due to the efficiency and robustness in tissue clearing. Clearing based on organic solvents consists of dehydration steps followed by delipidation and RI matching in the range of 1.56. Improvements on the initial BABB protocol led to the development of the three-dimensional imaging of solvent-cleared organs (3DISCO) protocol\(^1\)\(^9\). By replacing ethanol with tetrahydrofuran (THF) and using dibenzyl ether (DBE) instead of BABB, this protocol increased the transparency of the tissue (especially complex tissue such as the central nervous system) and reduced fluorescence quenching. This allowed the study of larger, more complex organs such as adult mouse brains. Several issues remained though. For instance, reports of anisotropic tissue shrinkage caused by the 3DISCO protocol hindered the ability to reconstruct or map areas of interest in 3D. To address this, Renier and colleagues developed the iDISCO+ protocol, which consisted of gradual dehydration of the sample in methanol followed by delipidation and RI matching in DBE\(^1\)\(^0\). This protocol significantly reduced the extent of tissue shrinkage. Altogether, Renier and colleagues could develop a mapping...
The loss of endogenous fluorescence remained a major hurdle for clearing protocols based on organic solvents. To this end, Pan et al. proposed the ultimate DISCO (uDISCO) protocol combining tert-butanol dehydration and RI matching in a cocktail of BABB, diphenyl ether, and vitamin E. The authors reported endogenous fluorescence pre-servation for up to 4 weeks. However, this protocol remained challenging due to i) the toxicity of the reagents, ii) the need to carry out the reactions at 35–40 °C, and iii) the need to fully perfuse the mice for several days. Although the effects of both temperature and pH on fluorescent proteins had previously been reported in the literature, none of the publications connected these effects with organic-solvent clearing protocols. To address this, the group of Matthias Gunzer developed an ethanol-based clearing protocol followed by RI matching in ethyl cinnamate (ECi). ECi is a compound approved by the US Food and Drug Administration, and therefore biosafety is an interesting feature of this protocol. In addition, by adjusting the pH of ethanol, Klingberg et al. maintained endogenous fluorescence for up to 14 days. However, ethanol-based dehydration provides only a moderate dehydration method. Therefore, ECi-based clearing should be chosen for samples that can be cleared easily.

Since then, Masselink et al. have generated a second-generation ECi protocol based on pH-adjusted propanol. It should be noted that the RI of ECi closely matches that of DBE or BABB (ECi: 1.558; BABB: 1.559; DBE: 1.562). We have observed that following ethanol-based clearing protocols, the samples can be imaged successfully in ECi for up to 3 hours (data not shown). Recently, Qi et al. proposed an alternative protocol for the preservation of endogenous fluorescence by altering pH and temperature of THF, the DISCO with superior fluorescence-preserving capability (FDISCO). The authors reported eGFP maintenance for up to 7 months. However, a major drawback exists when modulating the temperature of organic-based clearing: Storage of samples at 4 °C will lead to condensation in the vials and thus result in the presence of water which can re-opacify the sample.

To bypass the necessity of endogenous fluorescence preservation, several groups, including ours, developed immunolabeling protocols compatible with tissue clearing. These protocols closely resemble classical histological immunostaining with the main difference being antibody penetration. Espinosa et al. carried out multiple freeze-thaw cycles in methanol prior to immunolabeling. Renier et al. developed a bleaching step (removal of hematomas) together with the use of heparin and glycine and reported an increase in the signal-to-noise ratio. Belle et al. increased antibody penetration by i) combining permeabilization reagents (Triton X-100 and saponin) and ii) increasing antibody mechanics (incubation at 37 °C). More recently, the group of Ali Ertürk has developed a perfusion-assisted protocol, the so-called nanobody (VHH)-boosted 3D imaging of solvent-cleared organs (vDISCO). This protocol comprises intracardial perfusion of Atto/Alexa Fluor®–coupled nanobodies to enhance endogenous fluorescent epitopes by a factor of 1–2. Nanobody-driven immunostaining will be discussed later.

Although, initially, organic-based clearing has been implemented in laboratories more easily than aqueous-based clearing, recent development in aqueous-based protocols has quickly leveled the two techniques in regard to their feasibility, reproduducibility, and efficiency. In addition, aqueous-based processes have the advantage of biosafety and endogenous fluorescent protein maintenance. Two main principles exist in aqueous-based clearing, hydrogel-based clearing and RI matching using low RI media. Initial drawbacks of aqueous tissue clearing were either i) high transparency only after long incubation times, ii) costly procedures, or iii) high transparency only with small sample size (1 cm³ slices). To address these main issues, the group of Ueda deconstructed the clearing process into four main steps: decalcification, decolorization, delipidation, and RI matching. They then developed a screening strategy to identify major functional groups required in hydrophilic compounds to achieve each critical step. Over 1,600 chemical compounds were screened. Amino alcohols resulted in most efficient delipidation and decolorization, whereas aromatic amides provided best RI matching. Taken together, Tainaka and colleagues provided four novel clear unobstructed brain imaging cocktails and computational analysis (CUBIC) protocols for: i) mouse organs, ii) whole mouse or bones, iii) human tissues, and iv) complex tissues. To date, recent CUBIC protocols have been used on multiple organs as well as whole mouse.

In addition to hydrophilic aqueous solutions, hydrogel-based clearing protocols have provided another platform for robust tissue clearing. The first hydrogel-based clearing method, named CLARITY, depended on the perfusion of the organ with hydrogel monomers and subsequent heating for polymerization. This resulted in a scaffold that covalently cross-linked to protein in the tissue. The remaining “undesired” tissue was then removed using electrophoresis or a passive SDS bath. The major issue with this protocol was the implementation of the technique in non-expert laboratories. Although this technique became commercialized, the cost to carry out the protocol was incredibly high. To address these issues, several protocols were developed by the Gradinaru group at Caltech. The passive clarity technique (PACT) protocol decreased the hydrogel matrix concentration, which noticeably reduced the duration of the clearing procedure (~3 days for an adult mouse brain). However, reducing the hydrogel matrix concentration turned out to be a double-edged sword as it resulted in incredibly fragile tissues that became very difficult to image or handle. In addition, Yang et al. proposed the perfusion-assisted agent release in situ (PARS) protocol. In contrast to passive clearing, this protocol took advantage of the vasculature to perfuse the clearing agents, for time efficiency as well increased delivery. Here, the problem became the difficult implementation of large-scale clearing.
By making use of hydrogel technologies, the Boyden group devised a protocol for polymer scaffolding that could swell when subjected to hyper-hydrating solutions. This neat technique allowed for the first time the visualization of nanometer resolution and propelled the field forward towards super-resolution imaging. Recently, the Ueda group has developed a CUBIC protocol compatible with expansion microscopy to expand the volume of the whole mouse brain by 10-fold and thus reach single-cell resolution.

Decolorization of a sample is critical since it helps to harmonize the color of the tissue. In addition, certain tissues possess concentrated coloration which blocks the laser path even following clearing, for example the retinal pigment epithelium or the melanin present in the skin. The group of Hans Ulrich Dodt has recently published a decolorization protocol for Drosophila melanogaster clearing, termed Flyclear. This protocol stems from the first-generation CUBIC protocols, except for a change from Quadrol to 2,2',2'',2'''-ethylenedinitrilo-tetra-ethanol (THEED). The group of Ueda also proposed an efficient decolorization protocol using N-alkylimidazole. However, this protocol was specifically designed to decolor heme-rich tissues. Until now no protocol has been able to completely decolor the heavily pigmented mouse retinal pigment epithelium. To address this major void in the literature, we have developed a protocol which allows the clearing of the entire pigmented eye (manuscript in preparation). This protocol is compatible with multiple organic-based solvents, iDISCO+, 3DISCO, and ECi clearing. We have also attempted to adapt our protocol to aqueous-based clearing. However, the eye is composed of heterogeneous tissues which vary in composition, from the very dense sclera to the much softer retina. Clearing with aqueous solutions results in expansion of the tissue and strong deformation, and therefore, such techniques are not appropriate for clearing the entire intact eye. Our protocol provides the opportunity to remove highly concentrated melanophores from tissues and therefore provides a protocol that could clear difficult tissues such as highly vascularized samples or highly pigmented tissues such as skin.

Within the immensity of tissue clearing protocols available, new users are often left with the dilemma of which one to choose. Here the key is always the precise biological question one is attempting to answer. There is no universal best choice, because each protocol has its own advantages or disadvantages.

**Detection of endogenous proteins – the last limiting step**

The combination of advanced LSFM coupled to efficient and robust tissue clearing techniques now pave the way for solving complex biological questions. Although several protocols help to preserve endogenous fluorescence, we are currently restricted to only few fluorescent reporters. In addition, the expression of fluorescent reporters is restricted to transgenic models, and thus limits the molecular analysis of other species. Therefore, the development of new strategies for molecular targeting are required. Here, we will describe novel protocols and future possibilities.

A major approach for molecular targeting is antibody labeling. However, the main hurdle for whole-mount immunolabeling remains the passive diffusion of large antibody complexes across biological samples. In addition, antibody penetration across the entire sample is not always achieved, depending on tissue properties and size. To increase the rate of antibody labeling, several approaches are conceivable: exposing the sample to an electrical current or subjecting the sample to hydrodynamic pressure, thus driving convective flows across the sample. However, both approaches would damage soft and fragile tissues. In this effort, Kim et al. proposed a stochastic electrotransport protocol to increase the rate of antibody diffusion. The authors theorized that by applying a rotational electric field around a biological sample, only freely available charged molecules would be displaced, whereas cross-linked molecules would be unaltered. Their protocol enabled labeling of an 8 mm × 7 mm polymer using BSA–FITC in under 3 hours. In addition to immunolabeling, the authors also reported increasing rates of tissue clearing.

More recently, the group of Erturk proposed the vDISCO protocol to amplify the signal of endogenous fluorescent proteins by perfusing fluorescently coupled nanobodies (V₃H). To this day, the use of V₃H in whole-mount immunostaining is poorly studied. In comparison to traditional primary and secondary antibody complexes (>300 kDa), coupled nanobodies are more than 20-fold smaller (12–15 kDa). This provides a major step towards effective antibody diffusion and penetration across complex biological samples. Moreover, fluorochrome-conjugated nanobodies bypass the need for labeling with secondary antibodies and thus provide an invaluable gain of time. However, to date the usage of passive whole-mount immunostaining with nanobodies has not been tested. Another area of advancement will be the generation of more robust and photostable fluorescent dyes. Currently available dyes, i.e., Atto and Abberior® STAR dyes, possess high fluorescence quantum yields and decay times. They have been heavily used in super-resolution microscopy, which demands robust and bright dyes. Their use in whole-mount immunostaining coupled to tissue clearing however has been very little studied.

One major hurdle when it comes to tissue processing, be it sectioning or tissue clearing, is the finality of the process. Whilst several groups have focused on optimizing the antibody labeling or imaging quality, little efforts have been made towards maximizing the usage of a given biological sample. To that end, we have developed a protocol, multiple round DISCO (MR-DISCO) (manuscript in preparation), that allows: i) sectioning and imaging of cleared tissues at high resolution, ii) sectioning and targeting of mRNA, and iii) multiple rounds of immunolabeling of 3D tissue. This provides for the first time the possibility to reuse a sample and potentially describe highly complex structures in a single specimen (fig. 2). Whilst these developments provide a huge step forward in the field, there are certain limitations to this process. Consecutive rounds of clearing lead to a progressive shrinkage of the sample. Therefore, the development of software that enables adaptive measurement of tissue shrinkage must be developed to fully merge consecutive rounds of imaging into a single individual atlas.
Figure 2: Multiple-Round DISCO, a novel tissue processing workflow. MR-DISCO provides the ability to carry out a classical whole-mount immunostaining followed by tissue clearing, which can then be reversed by re-hydrating the sample and immunostaining again. This process can be coupled to tissue clearing, as shown in panel 1, or be coupled to cryosectioning for either immunohistochemistry (panel 2) or in situ hybridization (panel 3). Panel 1 shows an embryonic limb labeled for PAX7 (muscle precursors, magenta), and SOX-10 (Schwann cell precursors, green). Following re-hydration (MR-DISCO round 2), the same sample was immunostained for myosin heavy chain (muscle fibers, magenta), and choline acetyl transferase (motor axons, green). Panels 2 and 3 show sections from embryos first processed with MR-DISCO and then cryosectioned. For panel 2, brain sections were then immunostained for DAPI (nuclear marker, blue), PECAM-1 (blood vessel marker, green), tyrosine hydroxylase (dopaminergic and noradrenergic neurons, magenta), and β-III tubulin (axonal marker, green). For panel 3, spinal cord sections were hybridized with probes for ROBO3 mRNA (commissural neurons) and netrin 1 mRNA (floor plate and ventricular zone).
Outlook

Tissue clearing rose to fame in the past few years thanks to a century of optimizations in: i) optical devices such as LSFM, ii) robust, bright, and stable dyes, and iii) tissue clearing reagents. This interdisciplinary field is fast moving and has indisputably become central to biological research.

Protein targeting has been made possible thanks to multiple protocols adapting immunolabeling towards tissue clearing techniques. In recent years, novel technologies have allowed us to visualize multiple RNAs using small molecule fluorescent in situ hybridization (smFISH)\(^{37,38}\). Furthermore, the development of multiplexed in situ hybridization (MERFISH) and seqFISH provide an unlimited targeting of RNA in tissue sections\(^{39,40}\). Coupling this with tissue clearing techniques could give us access to data on single-cell RNA expression and localization in entire organs. Towards this goal, the group of Deisseroth developed a spatially-resolved transcript amplicon readout mapping (STARmap). The authors could amplify over 1,000 genes in cortical slices. Combinations of molecular analysis (MERFISH), cellular lineage labeling (brainbow), and tissue clearing could deliver data on molecular identity through to cellular morphology. Therefore, the development of tissue clearing techniques combined with molecular targeting approaches is at the forefront of the field.

In regard to public health, it is without a doubt that tissue clearing technologies will lead to breakthroughs in the fields of embryology\(^{41}\) or cancer biology\(^{31,32}\). For instance, the development of 3D tissue clearing protocol applicable to cancer biopsy samples will provide an immensely more comprehensive picture to pathologist than current techniques.

References

MACSima™ Imaging Platform provides new insights into cancer biology and target discovery by cyclic immunofluorescence–based imaging

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Analysis of cancer cell diversity and immune contexture based on protein expression is of high relevance for tumor subclassification and the development of novel targeted immunotherapies. However, these kinds of analyses are currently hampered by the lack of technologies that allow for sensitive and comprehensive multiplexed protein analysis. The novel MACSima™ Imaging Platform enables high-content, cyclic fluorescence–based imaging of individual biological samples in a fully automated fashion. We demonstrate how this novel platform can be used to analyze, for example, ovarian tumor and glioblastoma samples in research towards the discovery of potential targets for immunotherapy.

MACSima™ Imaging Platform – breaking the limits of antibody multiplexing in microscopy

Microscopy has made major progress in the last decades towards higher resolution, sensitivity, and tissue penetration, in particular by two-photon and light sheet microscopy. A further demand has been to increase the number of detectable targets on a single specimen. In standard fluorescent microscopy, multiplexing is restricted due to the range of excitation and emission spectra of fluorescent dyes and the limited spectrum of visible light. Increasing the number of dyes imaged simultaneously, ultimately leads to an overlap of spectra which – depending on the resolution – cannot be properly compensated. Therefore, many new technologies, either using optical or mass spectrometric readouts, have been introduced, including multi-epitope-ligand cartography (MELC)¹, ChipCytometry²−³, mass cytometry⁴, multiplexed ion beam imaging (MIBI)⁵, cyclic immunofluorescence (CycIF)⁶, multiplex immunohistochemistry⁷, co-detection by indexing (CODEX)⁸, Digital Spatial Profiling (NanoString®), NanoString Technologies Inc.), or InSituPlex® Technology (Ultivue Inc.).

Based on the MELC technology we have developed a new imaging platform for cyclic immunofluorescence analysis, introducing innovative solutions to address a number of shortcomings experienced with other technologies. The MACSima™ Imaging Platform provides a complete, integrated system including instrument (fig. 1A), optimized sample carriers, validated antibodies, and software. It operates by iterative fluorescent staining, image acquisition, and signal erasure, using multiple fluorochrome-conjugated antibodies per cycle (fig. 1B). Cyclic processing allows for the analysis of hundreds of different markers on a single sample.

The MACSima Imaging System includes optics (epifluorescence microscope, sCMOS camera, five emission filters, autofocus, six LEDs, three lenses), fluidics, mechanics (100 nm stage resolution), and data storage. The system allows fully automated processing of eight microscope slides or two 24-well imaging plates with hundreds of fluorochrome-conjugated antibodies or antibody fragments in a single run. Specially developed sample carriers, the MACSwell Imaging Frames, provide one, two, or four cavities and are mounted on standard microscope slides. MACSwell Micro Slides contain hundreds of thousands of small cavities that exactly fit one cell. Based on these sample carriers the system can process solid tissue slices, adherent cells, and suspension cells.

Two types of proprietary conjugates have been qualified for the analysis on the MACSima Imaging System: fluorochrome-conjugated REAfinity™ Recombinant Antibodies and REAlease® Releasable Antibodies. REAfinity Antibodies provide a higher lot-to-lot consistency and purity compared to mouse or rat hybridoma-derived monoclonal antibodies. All REAfinity Antibodies are recombinantly engineered using the same IgG1 isotype backbone. They do not require any FcR blocking step and thus are highly specific. REAlease Releasable Antibodies are a new type of antibody-fluorochrome conjugates, generated based on recombinantly engineered antibody fragments that individually possess low epitope binding affinities. A novel conjugation approach enables multimerization and fluorescent labeling of the fragments. The resulting complex can then bind to cell surface epitopes with high avidity. Subsequent disruption of the complex leads to release of the conjugates from the cell.
A plethora of REAfinity™ and REAlease® Antibodies coupled to FITC, PE, and APC are already available for use in combination with the MACSima™ Imaging System. Various antibody plates (REAscreen), each including hundreds of antibodies, were compiled for the analysis of acetone- or paraformaldehyde (PFA)-fixed or formaldehyde-fixed paraffin-embedded (FFPE) samples from human or mouse.

Finally, in-depth analysis of the image stacks generated on the instrument is achieved using a software developed in collaboration with Quantitative Imaging Systems. It allows an easy navigation through the hundreds of images and helps to identify markers and cell types of interest by segmenting images into single cells and clustering cells and proteins according to their profile across all cells. Multiple different clustering methods can be applied and visualized as scatter diagrams or back-plotted onto the microscopic image (fig. 1C).

**Comprehensive cyclic immuno-fluorescence analysis reveals new target candidates for chimeric antigen receptor–expressing T cells**

The advent of chimeric antigen receptor (CAR) T cell technology has opened new perspectives in the fight against cancer. CAR T cell–based therapies have resulted in a remarkable success in the treatment of hematopoietic malignancies⁹,¹⁰, but have not yet led to a breakthrough in eliminating solid tumors. Besides numerous tissue-immanent obstacles hampering T cell infiltration, such as the tumor microenvironment, part of the problem is also the lack of proteins that are suitable as targets for CAR T cells.

When considering a protein as a target for immunotherapy the coverage of tumor cells is predictive for its efficacy while the on-target/off-tumor toxicity is a potential threat. On-target/off-tumor toxicity is mainly based on the expression of tumor-associated antigens in healthy tissues under physiological conditions. Currently, most prediction methods for on-target/off-tumor expression are based on single-cell or even bulk mRNA expression data on healthy tissue. These models, however, have limitations, mainly due to poor predictability of the correlation between RNA and protein levels.

A number of cell surface markers are currently being investigated in CAR T cell therapy for solid tumors such as mesothelin (MSLN), ErbB2 (HER2), EGFR/EGFRvIII, GD2, CEA, IL-13Ra2, MUC1, FAP, PSMA, and PSCA.¹¹

We have used the MACSima Imaging Platform to analyze the toxicity profile of some of these markers and to screen for alternative markers. To that end, we analyzed a variety of tumor samples including glioblastoma, ovarian and pancreatic cancer and re-assessed potential markers for their expression profile in healthy tissue. Two studies exemplifying the power of the technology are discussed below.
Evaluation of the specificity and tissue distribution of MUC1

MUC1 (also termed mucin-1, a transmembrane glycoprotein) is an example of a marker which is aberrantly up-regulated in many types of cancer and is under evaluation in clinical trials as target for CAR T cells. It is also well established that mucins line the apical surface of epithelial cells in the lungs, stomach, intestines, eyes, and several other organs. To better understand the uniformity of MUC1 expression on tumor cells, we analyzed several patient-derived high-grade serous ovarian carcinomas using the MACSima™ Imaging Platform. Figure 2 shows a subset of the multi-parameter expression analysis of MUC1 and markers indicative of cell lineages and proliferation status, i.e., CD326 (epithelial), Ki-67 (proliferation), CD31 (endothelial), CD90 (stromal), and CD45 (differentiated hematopoietic cells). MUC1 was expressed on all analyzed ovarian carcinoma samples and, based on the co-expression with CD326 (EpCAM), it could be assigned to epithelial cells. This corroborates previous reports and suggests MUC1 as a potential target. However, the percentage of MUC1-positive cells among the different samples was highly variable. Moreover, MUC1 did not cover the complete population of CD326-positive cells in these tumors, which questions the selectivity of MUC1 as a CAR T cell target. Similar results were obtained for pancreatic ductal adenocarcinoma (not shown).

Next, we assessed the expression of MUC1 in healthy human tissues, i.e., kidney, colon, heart, lung, skin, and breast. As shown in figure 3, analysis of MUC1 compared to different lineage markers, i.e., CD326 (epithelial), CD29 (diverse), vimentin (mesenchymal), CD105 (endothelial, stromal), and CD90 (stromal) revealed that MUC1 was expressed on epithelial cells in different healthy human tissues. This finding not only stresses the importance of pre-clinical target validation on healthy tissues to predict potential on-target/off-tumor toxicity but also indicates which tissues and cell types could be affected when using MUC1 as target in CAR T cell therapies.

Characterization and classification of glioblastoma multiforme for the identification of new glioblastoma-specific markers

Glioblastoma multiforme is a highly malignant, incurable type of brain tumor. Current standard-of-care includes radiation, chemotherapy, and surgical resection when possible. Despite advances in each of these treatment modalities, survival rates for pediatric and adult high-grade glioma patients has remained largely unchanged over the course of several years. To identify new glioblastoma markers, we performed a two-step screening approach. First, patient-derived glioblastoma xenografts were dissociated into single cells using the Tumor Dissociation Kit, human and the gentleMACS™ Octo Dissociator with Heaters. The Mouse Cell Depletion Kit was applied to remove contaminating mouse cells and to obtain a pure xenograft cell suspension. Cells were analyzed by flow cytometry for cell surface marker expression using 523 fluochrome-conjugated antibodies (MACS® Marker Screen, human and additional 152 antibodies).

Figure 2: MUC1 shows epithelial expression as well as inter- and intratumor heterogeneity. Multi-parameter immunofluorescence-based imaging of six patient-derived high-grade serous ovarian carcinoma samples was performed on the MACSima Imaging System to assess co-expression of MUC1 with different lineage markers, i.e., CD326, Ki-67, CD31, CD90, and CD45. MUC1 is shown in red, DAPI in blue, and the indicated markers in green.
A ranking was applied according to the percentage of positive cells and the stain index, leading to a selection of 96 markers. In a second step, these markers were used for immunohistochemical characterization of primary glioblastoma samples using the MACSima™ Imaging System. To this end, fresh frozen cryosections of different glioblastomas were fixed with acetone. Each specimen was exposed to 96 fluorescently labeled antibodies by repeated cycles of antibody staining, image acquisition of each region of interest (ROI), and erasure of the fluorescence signal. The resulting 2D image stacks were segmented and single-cell imaging data were used for protein expression profiling and pattern recognition.

We classified the different primary glioblastoma tumors based on the expression of PDGFRα, p53, synaptophysin, CD44, nestin, podoplanin, GFAP, and EGFR and following a classification scheme published by Motomura et al.\textsuperscript{15}

Figure 4 shows representative immunofluorescence stainings of two glioblastoma samples and their classification as either mixed type (fig. 4A), characterized amongst others by a high expression of EGFR, or as astrocytic/mesenchymal type (fig. 4B), which is characterized by strong expression of GFAP, podoplanin, CD44, and nestin, moderate expression of p53 and PDGFRα and is negative for EGFR.

Figure 3: MUC1 is expressed in epithelium of healthy human tissues. Multiparameter immunofluorescence-based imaging of healthy human kidney, colon, heart, lung, skin, and breast tissue was performed using the MACSima Imaging System to assess co-expression of MUC1 with different lineage markers, i.e., CD326, CD29, vimentin, CD105, and CD90. MUC1 is shown in red, DAPI in blue, and the indicated markers in green.

Figure 4: Classification of primary glioblastoma tumors. Immunofluorescence-based imaging was performed using the MACSima Imaging System. Representative immunohistochemical stainings of two glioblastoma samples are shown. According to the classification by Motomura et al.\textsuperscript{15} the sample shown in (A) is classified as mixed type due to high expression levels of EGFR. In contrast, the sample in (B) belongs to the astrocytic/mesenchymal type, which is characterized by strong expression of GFAP, podoplanin, CD44, and nestin, moderate expression of p53 and PDGFRα, and is negative for EGFR. EGFR is shown in red, nestin in green, p53 in cyan, and DAPI in blue.
Next, we analyzed the expression of well-established glioblastoma markers used in CAR T cell–based clinical trials, such as ErbB2 (HER2) and IL-13Rα2 (A). Screening of 96 antibodies led to the identification of new marker candidates (A). Graphs show average mean fluorescence intensities (MFI) per region of interest (ROI) of the known glioblastoma markers and the new marker candidates (B), and the percentage of positive cells, i.e., cells per ROI with an MFI above background (C). Lines indicate mean values.

**Conclusion and outlook**

In summary, we introduce a novel, fully integrated and automated cyclic immunofluorescence imaging platform that can be used to analyze a broad variety of specimens with an unprecedented number of antibodies. This technology, which is tailored to maximize the information obtainable from valuable tumor biopsy material, opens up new perspectives for understanding the complexity of tumors and will have a deep impact on the study of tumor stratification and the development of new therapies. Integration of the single-cell protein profiles derived from this technology with single-cell sequencing and 3D microscopy data will be the next steps to expand our knowledge on healthy and diseased cells even further.

**References**


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From graft engineering to lymphocyte engineering in high-risk pediatric leukemia

Hematopoietic stem cell transplantation (HSCT) is a well-established clinical procedure. Invented over 50 years ago as a way to replace the hematopoietic and lymphoid compartment after destruction of patient bone marrow with high-dose radiotherapy, it evolved over the years into a wide family of sophisticated therapies. This article provides insight into the different strategies and perspectives.

Hematopoietic stem cell transplantation – benefits and risks
Modern HSCT targets diverse diseases, from high-risk leukemia to inherited metabolic disorders to organ-specific autoimmunity. HSCT, as a complex medical technology, combines best practices from related fields which act synergistically to improve, year by year, the outcomes of the procedure.

Since its invention, HSCT has been fascinating us with its enormous potential to cure otherwise incurable diseases. However, HSCT also has frightening aspects with an innumerable array of acute and delayed complications, risk of death, and irreparable (permanent) disability. Despite the well-known risks of the procedure, every year thousands of patients around the world attempt this journey to take a chance, usually the only chance, to combat mortal disease.

Many years ago, it was realized that part of the therapeutic power of HSCT in leukemia resides in the compartment of donor T lymphocytes, which recognize the genetically encoded disparity between the donor and the recipient. The phenomenon of graft-versus-tumor response is one of the major therapeutic principles of the procedure. Biologically, the graft-versus-leukemia (GVL) effect of the graft is tightly associated with a much-feared complication – the so-called graft-versus-host disease (GVHD), which results from a damaging immune response of donor T and B lymphocytes towards host tissues. For decades the “central dogma” of HSCT was that donor T cells, transferred as part of the hematopoietic stem cell graft, cause GVHD and mediate the GVL effect. One of the direct consequences of this dogma is that any action, aimed at restricting the activity or content of the grafted T cells, be it pharmacological immunosuppression or ex vivo or in vivo T cell depletion, will decrease both GVHD (favorable effect) and GVL (unfavorable effect). In practice, ex vivo T cell depletion was known to be very effective in preventing clinical GVHD, but stringent removal of T cells also resulted in an increased risk of relapse and heightened the risk of severe infections, even in the absence of pharmacological immunosuppression.

Beyond the vast array of biological and practical issues of HSCT, one point is often neglected: For many years this curative therapy was simply not available to most patients in need. One reason for restricted access was that HSCT was a complicated and expensive technology. However, even in settings of ample resources many patients were denied access to HSCT due to another key barrier: donor availability. Since the early years of HSCT, physicians knew that mismatch of histocompatibility antigens between the donor and the recipient determine the frequency and severity of GVHD. This led to the common practice of searching for a “perfectly” matched donor. Although based on very strong observations, this practice is suboptimal for at least two reasons: First, the prolonged search process leads to exclusion of patients with more aggressive forms of leukemia and severe infections, creating a certain bias for further analysis. Second, patients representing ethnic minorities and those with rare HLA haplotypes have only a slim chance for identification of a suitable donor. Ultimately, this practice leads to an inadequate medical care of a significant proportion of patients. The path towards systematic change of this practice was led by early work of a group in Perugia, Italy, who demonstrated that transplants from full haplotype-mismatched donors can be successfully used in patients with leukemia.

Early studies
Early work of Aversa et al. demonstrated that transplantation of a high dose of purified CD34+ cells overcomes the histocompatibility barrier and allows for engraftment of full
haplotype-mismatched hematopoiesis. It was established that the problem of GVHD can be largely solved without post-transplant immunosuppression. This work also uncovered the fundamental role of NK cells in the post-transplant control of leukemia in the setting of haploidential HSCT. Transplantation of purified CD34+ cells was not without technical obstacles and risks. The technology required use of intensive preparative regimens and “megadose” (>10 million cells per kg) of stem cells and was associated with delayed immune recovery and fatal infections. Despite these problems, this experience produced key evidence that haploidential transplants can be used on a systematic basis and that this type of stem cell graft may produce clinically relevant outcomes. Transplantation of highly purified positively selected hematopoietic progenitor cells paved the way for further development of graft manipulation technologies, which are briefly discussed below.

**CD3+/CD19+ cell depletion**
Discovery of the outstanding role of donor NK cells in the control of leukemia was probably the starting point for development of the next generation of graft engineering approaches, aiming at preserving the NK content of the graft, while depleting the effectors of GVHD, i.e., T cells, from the graft. Simultaneous depletion of CD3+ and CD19+ cell populations was developed by Rupert Handgretinger’s group in Tübingen, Germany to keep GVHD under control (by T cell depletion) and prevent Epstein-Barr virus (EBV)-associated lymphoproliferation (by depleting the B cell reservoir of EBV). Clinical development of CD3+/CD19+ cell depletion led to a significant improvement of the engraftment potential, enabling stable graft function after a significantly reduced intensity of the conditioning regimen. It was also shown that transplantation of haploidential CD3+/CD19+ cell–depleted grafts led to improved early immune recovery. One major drawback of the approach was that the efficiency of T cell depletion was 1 log lower than with positive selection of CD34+ cells, which was associated with less stringent prevention of GVHD. The success of the approach was also partly dependent on accurate application of in vivo blockade of CD3+ cells with OKT3, a medication of limited availability, which was later withdrawn from the market.

**TCRαβ+ T cell depletion**
As a continuation of CD3+/CD19+ cell depletion, the technique of TCRαβ+ T cell depletion was developed in the mid 2000’s and introduced into clinical practice around 2011. This platform was built on the same general idea of keeping as much as possible of the beneficial cell populations within the graft, while effectively depleting the compartment of GVHD effectors. With this approach, not only the high dose of NK cells remains intact, but also a population of TCRγδ+ T cells becomes part of the final product. TCRγδ+ T cells were shown in multiple studies, and in vivo model studies to possess significant anti-tumor and anti-viral activity, while being unable to induce GVHD. An important technical feature of double-labeling significantly improved the quality of depletion of the undesired cell population, making the procedure at least as effective as positive selection of CD34+ cells, improving the recovery of hematopoietic stem cells to >85%.

Depletion of both TCRαβ+ T cells and CD19+ B cells was tested in multiple single-center studies, mostly among children with high-risk acute leukemia. It was shown that use of this technology leads to very robust and rapid engraftment. More intensive preparative regimens are associated with >95% primary engraftment rate, while reduced intensity regimens may lead to 15% primary graft failure, which can be overcome by administration of a second graft in most of the cases. Control of acute and chronic GVHD was good in most reports, with best results achieved when rabbit anti-thymocyte globulin (ATG) was used proximally to the graft infusion. In our experience the use of horse ATG was associated with a somewhat higher incidence of both acute and chronic GVHD, although the rate of severe, grade III-IV GVHD was low. Recovery of T cells was improved in comparison to the previous generation of graft manipulation. It was especially notable that significant recovery of TCRγδ+ T cells could be detected as early as day +15 after grafting. Recovery of TCRαβ+ T cells was quantitatively improved, but for up to 6 months after transplantation peripheral blood TCRαβ+ T cells were derived from oligoclonal expansion of a few clones, most probably derived from non-depleted residual TCRαβ+ T cells of the graft. De novo production of naive post-thymic donor TCRαβ+ T cells gradually emerged after day +120 and achieved a normal level after one year. The TCR repertoire largely reflected this pattern with broad protective TCR repertoire formation by the end of the first year after HSCT.

**Figure 1:** Recovery of major lymphocyte populations over time after transplantation of TCRαβ+ T cell- and CD19+ B cell–depleted grafts. Numbers on the y-axes indicate the absolute cell count per mL of peripheral blood.
Outcome of HSCT with TCRαβ+ T cell– and CD19+ B cell–depleted transplants, among children with acute leukemia, is that the outcomes of haploidentical transplantation are not at all inferior to the outcomes of transplantation using grafts from matched unrelated donors. The direct consequence of this finding is that application of this type of graft manipulation transforms HSCT from a therapy for the lucky people into a universally and timely available treatment option. We believe that this transformation will have an important practical impact on the use of HSCT and donor choice in the near future. Despite the significantly improved results of haploidentical transplantation with TCRαβ+ T cell–depleted grafts, delayed recovery of adaptive immune response led to high frequency of virus replication. Moreover, up to 10% of the recipients succumbed to transplant-related mortality, in most cases caused by viral infections, including cytomegalovirus (CMV) and adenovirus. EBV rarely caused severe lymphoproliferation due to effective ex vivo depletion of B cells. Among those recipients who also received in vivo B cell depletion by rituximab, EBV-related problems after haploidentical HSCT were almost eliminated. Over the years the need for improvement of pathogen-specific immune reconstitution was realized by many in the field and became the driving force for further development of graft engineering.

**CD45RA+ cell depletion**

CD45RA+ cell depletion was developed as a graft engineering approach based on seminal research, which demonstrated that effectors of GVHD reside predominantly in the fraction of naive T cells. Animal research was corroborated by human in vitro studies, which showed that the frequency of alloreactive T cells is 1 log lower in the fraction of memory T cells and that broad pathogen reactivity is retained during active disease still had a chance of survival over 50% (figs. 2 and 3).

When tested among different cohorts of children with acute leukemia, transplantation of haploidentical TCRαβ+ T cell–depleted grafts resulted in long-term survival of over 70% of the recipients. Most of the survivors enjoy a high quality of life due to very low incidence of chronic GVHD. Interestingly, among children with acute lymphoblastic leukemia (ALL), optimal results (75% survival) were achieved with total body irradiation (TBI)–based preparative regimen, while among those conditioned with chemotherapy the 3-year overall survival appeared significantly lower, at around 50%.

Among the patients with acute myeloid leukemia (AML), survival was dependent on the remission status at the moment of transplantation. Patients who received the graft in complete remission enjoyed long-term survival of over 80%, while patients treated during active disease still had a chance of survival over 50% (figs. 2 and 3). These promising results were achieved among children with AML with reduced toxicity, non-TBI preparative regimen, which included treosulfan with either melphalan or thiopeta. Our group has been using the platform of TCRαβ+ T cell depletion to process grafts both in the setting of haploidentical and matched unrelated HSCT. One of the major practical (although retrospective) findings, based on over 400 procedures in children with acute leukemia, is that the outcomes of haploidentical transplantation are not at all inferior to the outcomes of transplantation using grafts from matched unrelated donors.

**Figure 2:** Outcome of HSCT with TCRαβ+ T cell– and CD19+ B cell–depleted transplants, among children with acute myeloid leukemia, grafted in complete remission. TRM: transplant-related mortality; CIR: cumulative incidence of relapse; EFS: event-free survival; OS: overall survival. Graphs in red correspond to matched unrelated donors (MUD), graphs in blue to haploidentical donors (Haplo).

**Figure 3:** Outcome of HSCT with TCRαβ+ T cell– and CD19+ B cell–depleted transplants, among children with chemorefractory acute myeloid leukemia, grafted in active disease from haploidentical donors. TRM: transplant-related mortality; OS: overall survival.
Figure 4: Outcome of low-dose memory (CD45RA+ cell–depleted) donor lymphocyte infusion (DLI). (A) Cumulative incidence of de novo acute GVHD after low-dose memory DLI. (B) Dynamics of the absolute count of CMV-specific T lymphocytes (CMV-T) in peripheral blood of the recipients of low-dose memory DLI. Numbers on the y-axes indicate the absolute count of CMV-reactive T cells per 300,000 MNCs.

After depletion of naive, CD45RA+ T cells by immunomagnetic labeling. As soon as a clinical-grade reagent for depletion of CD45RA+ cells became available, a series of clinical trials was initiated to test the safety and efficacy of this approach. In the first-in-human trial of using grafts engineered by CD45RA+ cell depletion in the setting of HLA-matched transplantation, it was shown that the incidence of acute GVHD (aGVHD) was not significantly reduced, while the incidence of chronic GVHD was remarkably low. Most cases of aGVHD were mild, and patients responded to steroid therapy. Remarkably, no cases of non-relapse mortality were registered among the younger patients. Application of CD45RA+ cell depletion in the setting of haploidentical transplantation in children led to mixed results in terms of aGVHD control, while outstanding results were achieved in immune reconstitution and non-relapse mortality. Beyond the use of CD45RA+ cell depletion as a basic graft manipulation procedure, it was also suggested that infusion of donor memory T cells can be used as a measure to improve pathogen-specific immune recovery. The basic idea is that the low relative frequency of the alloreactive T cells versus pathogen-specific T cells in the CD45RA+ cell–depleted product creates a window of opportunity to use donor lymphocyte infusions (DLI) to control infections without increased risk of GVHD. In order to systematically test this approach, we have developed a pilot trial to test the safety and potential efficacy of low-dose memory DLI after engraftment of the primary graft in the setting of TCRαβ+ T cell-depleted HSCT from haploidentical and matched unrelated donors. It was demonstrated that infusions of up to 100×10⁶ memory T cells in the context of haploidentical grafts and up to 300×10⁶ with matched unrelated grafts are safe and are able to initiate meaningful virus-specific immune responses (fig. 4). To further extend this approach we initiated a prospective trial of low-dose memory DLI co-infusion with the primary TCRαβ+ T cell–depleted HSCT. The goal was to test the safety of the approach in the setting of pre-engraftment infusion and efficacy to prevent reactivation of CMV. In order to execute this study, one important obstacle had to be bypassed: Infusion of rabbit ATG close to the day of transplantation creates unpredictable serum concentrations of polyclonal anti-lymphocyte antibodies, which may damage the memory T cells infused in small absolute numbers. To solve this problem we created a protocol, which substituted ATG with a combination of biologics, targeting pro-inflammatory cytokine IL-6 and the costimulatory CD28/B7 axis. Both tocilizumab and abatacept are being tested for prevention and/or therapy of GVHD, and we hypothesized that these agents will have minimal effects on memory T cell responses towards viral pathogens. In the preliminary analysis of this trial we were able to show that substitution of rabbit ATG with tocilizumab and abatacept is safe and does not diminish the control of GVHD, neither does it negatively affect engraftment. While the detailed analysis of the virus reactivation rate and the development of virus-specific immune responses is ongoing, it was rewarding to register an unprecedented, low transplant-related mortality of 2% in a cohort of >120 pediatric patients with malignant hematologic disease. These results suggest that even small absolute numbers of pathogen-specific T cells are able to provide effective protection from common infections after HSCT in the setting of minimal post-transplant pharmacologic immunosuppression and omission of serotherapy. The developed combination of TCRαβ+ T cell–depleted HSCT and CD45RA+ cell–depleted DLI in the context of reduced toxicity conditioning and precise immunomodulation solves several major questions of HSCT, namely, effective engraftment across HLA barriers, prevention of severe GVHD, and reduction of infection-related mortality. It is remarkable to note that effective prevention of GVHD is not accompanied by loss of the GVL effect, as reflected in a low incidence of leukemia relapse around 20%. Low non-relapse mortality and absence of post-transplant immunosuppression create a solid foundation for further improvement of HSCT outcomes. This improvement could be based on the integration of leukemia-specific agents into the program of HSCT therapy, in the form of targeted immunotherapy, vaccines, and small molecule inhibitors of key leukemia drivers.
CD19 CAR T cells and beyond

Effective targeting of surface antigens, such as CD19, on leukemia cells by genetically modified T cells expressing a chimeric antigen receptor (CAR), is a new reality of clinical hematolgy. A high rate of deep molecular remission, achieved with CD19 CAR T cells among adult and pediatric patients with chemorefractory ALL, constitutes a true breakthrough with far-reaching consequences to the way we will treat these diseases in the coming years. Putting aside the new universe of lymphocyte engineering and the seemingly endless opportunities to create therapeutic T cells with improved characteristics, two major strategic questions will have to be solved in order to make the best of the new technology. The first complex of questions relates to the optimal positioning of CD19 CAR T cell administration in the programmed therapy of B-ALL. Will it replace HSCT in a proportion of patients? What are the right triggers for and the best timing of HSCT as a post-CAR T cell consolidation option? Is it safe to move gene-modified T cells forward in the line of therapies and will it increase the chance that CAR T cells will be effective as an ultimate standalone therapy? The answer to these questions may only come from well-organized multicenter clinical trials, involving thousands of patients. This condition leads to the second major question: What is the most effective model of delivery of this type of therapy to the patients? Protocols of CAR T cell manufacturing have been significantly improved over time, and failures to produce the target product are usually the consequence of poor quality of the starting material, i.e., the patient T cells. T cell manufacturing at industrial-type cell factories is the predominant way of delivery for CD19 CAR T cell products currently available on the market. Point-of-care production is used in academic clinical trials and creates unique opportunities for flexible and timely delivery of the therapeutic cell product. An automated process for CD19 CAR T cell production in the closed system of the cell manufacturing platform CliniMACS Prodigy®, developed by Miltenyi Biotec, is a major step forward to make the sophisticated T cell manufacturing available to sites with less expertise in cell culture and clean room processes. In principle, a programmable closed-system process, which integrates the steps of magnetic cell separation, cell culture, and washing, holds the potential to lower the requirements for clean room environment, personnel, and ultimately the final cost of the CAR T cell product. Local production will obviate complicated logistics and may significantly facilitate the integration of CAR T cell–based therapy into the complex clinical care of patients with advanced acute leukemia. We have completed recently the recruitment into the first clinical trial of CD19 CAR T cells, locally produced on the platform of the CliniMACS Prodigy. Thirty patients, aged 2 to 22 years, with relapsed/refractory ALL were enrolled. In all but one case, manufacturing of the CD19 CAR T cell product was successful, the target dose of gene-modified cells was achieved, and the final product passed quality control in all cases. An over 95% success rate of manufacturing and a median time from apheresis to infusion of 10 days compares favorably to most published reports and sets a very high standard of robustness and quality for the field.

Prospects

As clinicians in the field of pediatric hematology/oncology we are used to face difficult cases, when all curative options are exhausted, and palliation replaces cure as a primary goal. An even more stressful scenario is a case when curative options do exist “in principle” but cannot be made available to the patient for a whole range of financial, logistical, and regulatory reasons. Closings this gap and making curative therapies universally available and affordable is a goal shared by many in the field. Clinical development of the new therapies and reincarnation of the old ones into more effective and safe models is more than ever dependent on the intensive collaboration of the industry and clinical researchers. The technological ecosystem created by Miltenyi Biotec is an example of visionary development of clinical bioengineering instruments and seems to be perfectly suited for this type of collaborative effort.

References


Unless otherwise specifically indicated, Miltenyi Biotec products and services are for research use only and not for therapeutic or diagnostic use. The CliniMACS System components, including Reagents, Tubing Sets, Instruments, and PBS/EDTA Buffer, are designed, manufactured and tested under a quality system certified to ISO 13485. In the EU, the CliniMACS System components are available as CE-marked medical devices for their respective intended use, unless otherwise stated. (The CliniMACS Reagents and Biotin Conjugates are intended for in vitro use only and are not designated for therapeutic use or direct infusion into patients. The CliniMACS Reagents in combination with the CliniMACS System are intended to separate human cells. Miltenyi Biotec as the manufacturer of the CliniMACS System does not give any recommendations regarding the use of separated cells for therapeutic purposes and does not make any claims regarding a clinical benefit. For the manufacturing and use of target cells in humans the national legislation and regulations – e.g. for the EU the Directive 2002/98/EC (“human tissues and cells”), or the Directive 2002/98/EC (“human blood and blood components”) – must be followed. Thus, any clinical application of the target cells is exclusively within the responsibility of the user of a CliniMACS System. In the US, the CliniMACS CD34 Reagent System, including the CliniMACS Plus Instrument, CliniMACS CD34 Reagent, CliniMACS Tubing Sets TS and LS, and the CliniMACS PBS/ EDTA Buffer, is FDA approved as a Humanitarian Use Device (HUD), authorized by U.S. Federal law for use in the treatment of patients with acute myeloid leukemia (AML) in first complete remission. The effectiveness of the device for this indication has not been demonstrated. All other products of the CliniMACS Product Line are available for use only under an approved Investigational New Drug (IND) application or Investigational Device Exemption (IDE). CliniMACS Microbeads are for research use only and not for human therapeutic or diagnostic use. In the US, the CliniMACS Prodigy® T Cell Transduction Process is available for research use only.)
Biophysical technology (FRET, FLIM) homes in on tumor cell biology – the epidermal growth factor receptor

In this communication, our focus is on the EGFR receptor (EGFR), a founding member of the receptor tyrosine kinase (RTK) family of transmembrane receptors (HER1, HER2, HER3, HER4) that collectively utilize flow cytometry and fluorescence microscopy with emphasis on FRET and FLIM. We anticipate that some of the considerations may be of interest to those familiar with EGFR signaling pathway biology.

Already in the 1970s and 80s, before the structure of EGFR was determined, it was apparent that the canonical EGFR signaling pathway, which begins with activation of its extracellular domain by binding of a ligand (e.g. epidermal growth factor, EGF), requires dimerization for activation of the kinase domain and downstream initiation of DNA synthesis and cell proliferation. Structural studies by X-ray crystallography⁴ and subsequent biophysical studies using fluorescently labeled EGF and antibodies (FRAP), FLIM, and other methods utilizing fluorescence Recovery After Photobleaching (FRAP), FLIM, and other methods utilizing fluorescence Recovery After Photobleaching (FRAP), FLIM, and other methods utilizing fluorescence Recovery After Photobleaching (FRAP), revealed that EGFR is a dimeric protein, with each subunit consisting of an N-terminal extracellular domain, a transmembrane domain, and a C-terminal intracellular domain containing a kinase domain.

In addition, it has been shown that EGFR exists in an auto-inhibited conformation. Ligand binding of EGF to EGFR results in receptor dimerization and auto-phosphorylation of the intracellular tyrosine-rich C-terminal tail (fig. 1). The phosphorylated EGFR dimer then interacts with adapter proteins, such as from biophysical measurements by Fluorescence Correlation Spectroscopy (FCS), Fluorescence Resonance Energy Transfer (FRET), and Fluorescence Lifetime Imaging Microscopy (FLIM). We anticipate that some of the considerations may be of interest to those familiar with EGFR signaling pathway biology.

**References**

Biophysical technology (FRET, FLIM) homes in on tumor cell biology – the epidermal growth factor receptor

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Förster Resonance Energy Transfer (FRET) and Fluorescence Lifetime Imaging Microscopy (FLIM) enable the detailed investigation of protein-protein interactions and conformational rearrangements, illustrated here in studies of the canonical EGFR receptor signaling pathway. In addition, we discuss aspects of methodological and probe design strategies for quantitative assessment of such a system, utilizing flow cytometry and fluorescence microscopy with emphasis on FRET and FLIM.

In this communication we first focus on the human epidermal growth factor (EGF) receptor (EGFR, also erbB1 and HER1), a founding member of the receptor tyrosine kinase (RTK) family, which is frequently mutated and/or upregulated in human tumors. EGFR is probably the most studied RTK, yet after more than two decades one is still deciphering new aspects of its multifaceted roles in normal physiology and in tumorigenesis. EGFR is a member of a family of transmembrane receptors (HER1, HER2, HER3, HER4) that collectively respond to at least 14 different ligands that regulate their function(s). After briefly summarizing current perceptions about the canonical EGFR signaling pathway, we shift to aspects of methodological and probe design strategies for quantitative assessment of such a system, utilizing fluorescence microscopy and flow cytometry with emphasis on Förster Resonance Energy Transfer (FRET) and Fluorescence Lifetime Imaging Microscopy (FLIM). We anticipate that some of the considerations may be of relevance to the broad panorama of evolving Miltenyi Biotec products and strategies.

The canonical EGFR signaling pathway

Already in the 1970s and 80s, before the structure of EGFR was determined, it was clear that EGF ligand activation of the EGFR and downstream initiation of DNA synthesis and growth required dimerization and/or higher degree of association of the receptor at the plasma membrane. This perception arose from studies of the binding of monoclonal antibodies and their Fab fragments as well as from biophysical measurements by Fluorescence Recovery After Photobleaching (FRAP), FLIM, and other methods utilizing fluorescently labeled EGF and antibodies applied to cells. Subsequently, structural studies by X-ray crystallography and supported by our FLIM measurements revealed that the quiescent, unliganded receptor exists in an auto-inhibited conformation. Ligand binding leads to rearrangement of the domains I and II, receptor dimerization, and autophosphorylation of the intracellular tyrosine-rich C-terminal tail (fig. 1). The phosphorylated EGFR monomer EGFR dimer

![Figure 1: Dimerization – central dogma of EGFR activation. Left: inactive; right: active. Adapted from ref. 9.](image-url)
amino acid sidechains are differentially recognized by numerous protein factors of parallel signaling pathways, mediating reversible phosphorylation and ubiquitination, and leading to recruitment and signaling at clathrin-coated pits, rapid endocytosis, vesicular trafficking, and ultimate downregulation by the protein degradation machinery of the cell.

Figure 1 represents only one, limited physical biochemical view of the EGFR system. A host of issues as well as methods for their resolution exist. They include: comparative monomer-dimer-tetramer activity and function; alternative dimeric conformations and other structural features inferred from crystallography, NMR, and molecular dynamics; role of transmembrane and submembrane domains; cell surface clustering; local membrane structure and interactions; trafficking and diffusion in the plasma membrane under the influence of the juxtamembrane cytoskeleton; and the role of EGFR in intracellular compartments.

**Receptor ligands differentially induce different signaling dynamics**

All of the above aspects define potential targets for diagnostic and therapeutic intervention. Ligand interactions in the tumor context are of particular interest with respect to signaling, inasmuch as the liganded and unliganded receptors exhibit distinct vesicular and phosphorylation dynamics. EGFR can bind seven different ligands which modulate the stability and recycling and downregulation of the receptor, as does heterodimerization with other members of the HER family with binding of seven additional ligands. The ligands are differentially expressed inasmuch as the liganded and unliganded receptors exhibit distinct vesicular and phosphorylation dynamics. EGFR can bind seven different ligands which modulate the stability and recycling and downregulation of the receptor, as does heterodimerization with other members of the HER family with binding of seven additional ligands.

The HER receptors are essential for embryogenesis, being expressed on embryonic stem cells in the epithelial and neuronal lineages, and thereby promoting division and migration. Many brain tumors as well as non-small cell lung cancer (NSCLC) and colon tumors express the antigenic cohort of these undifferentiated precursors. The Yosef Yarden group has proposed that somatic mutations of the HER receptors alone can promote cell growth as well as change the kinetics and function of the receptor by saturating the receptor binding sites with ligand. Tumors result in and/or arise from a disruption of organ homeostasis, involving alterations of the intimate intracellular communication that leads to dynamical solutions constituting what has been intriguingly termed a “cognitive” biochemical behavior. Homeostasis at the level of the organism adapts to changing conditions in the environment by promoting growth when nutrients are available or by using autophagy for maintenance during starvation. EGFR inhibits autophagy through the activation of downstream AKT but the unphosphorylated EGFR also mediates other pathways that promote autophagy, thus playing a key role in the maintenance of homeostasis. Various EGFR non-canonical pathways are also important in cellular responses to stress, which have been reviewed in relation to tumor therapy. In the early 1920s Warburg described the particular physiological mechanism by which tumor cells switch from oxidative phosphorylation to strong glycolysis in the presence of oxygen, resulting in increased lactate levels promoting angiogenesis, immune escape, cell migration, metastasis, and autonomous metabolism. An “inverse Warburg” effect has been recently invoked and serves to highlight new potential diagnostic and therapeutic targeting strategies.

**Profiling protein-protein interactions by FRET and FLIM**

Besides the essential role of proteomics, the merging of genetic, (bio)chemical, and imaging technology have contributed greatly to the elucidation of the EGFR system. Many insights have been detailed in a series of publications from the laboratory of Philippe Bastiaens (Max Planck Institute of Molecular Physiology, Dortmund). For example, the group has devised a FRET-based conformational EGFR indicator using genetic code expansion that reports on conformational transitions in the EGFR activation loop, revealing a catalytically active conformation in EGFR monomers that are operative at low ligand (EGF) levels. In another study, extrinsic, intrinsic, and effector caspase activities were also imaged by FRET, in this case by fluorescence anisotropy microscopy. A further very useful innovation is the technology for inducing reversible anisotropy microscopy. The combination of FRET and FLIM was featured in our very early and more recent studies of conformational rearrangements of EGFR in living cells.

The FRET-FLIM technology has been reviewed continuously and extensively; we refer the reader to a comprehensive monograph and to a more recent review of multiplexed time-resolved live cell imaging. We now introduce some concepts that in our estimation reinforce the utility of the FRET techniques but are not generally perceived in the FRET literature or incorporated in the design of FRET applications.
A fluorophore is a photonic “enzyme.” This notion was introduced in the first of three reviews on FRET imaging published by our group\(^{40–42}\). It is demonstrated that just like an enzyme, a fluorophore exhibits a substrate (excitation light, \(\lambda_{\text{exc}}\)); a linear dependence on (low) substrate concentration; a product (emission light, \(\lambda_{\text{em}}\)); a “\(V_{\text{max}}\),” \(k_f\); a “\(K_m\),” \((\sigma \tau)^{-1}\) (\(\sigma\) is the absorption cross-section and \(\tau\) the lifetime); reversible (quenching) and irreversible (photobleaching) inhibition; and competition such as FRET and singlet-to-triplet intersystem crossing. The enzyme analogy is very useful in devising new strategies for probe design and corresponding FRET measurements.

The quantitative manifestation of FRET is defined by a set of parameters subject to the influence of numerous processes (fig. 2, table 1), many of which (i.e. not only D-A separation, \(r_{DA}\)) can be exploited alone and in combination\(^{40–42}\), offering a myriad of possibilities.

The equations usually applied in data reduction incorporate the Förster constant \(R_0\), which includes as a factor the unperturbed donor quantum yield \(Q_D\), leading to the standard expression for the transfer efficiency \(E = 1 + (\sigma \tau / R_0)^2\). However, \(Q_D\) is often unknown or not even constant in a given FRET interaction, e.g., a binding reaction, such that the evaluation is often best conducted without invoking \(E\). In fact, the fundamental photophysics of the FRET phenomenon is stated as a relation between the transfer rate constant \(k_t\) and the radiative (emission) rate constant \(k_f\) (usually invariant) and not the intrinsic decay constant (reciprocal lifetime) of the donor. Thus, in a systematic catalog of FRET methods (table 2; see also ref. 40) it is useful to distinguish between those that necessarily involve the estimation of \(E\) and those that do not. The latter circumstance applies in many imaging situations based on stationary or time-varying signals involving the acceptor signals (±donor). Under these conditions, the following equation provides a very practical, useful estimation of FRET in terms of the ratio \(k_t / k_f\) and the indicated experimental quantity \(F\). The relationship applies to every relevant pixel in an image as well as to signals acquired with single detectors as in a flow cytometer (see footnote (a) on the next page).

**Figure 2:** Excited state transitions between donor (D) and acceptor (A). Adapted from ref. 41. Not shown is the thermal reverse T* to D* transition leading to delayed fluorescence.

**Table 1:** Ground and excited state properties and phenomena affecting FRET parameters.
Adapted from ref. 42. \(J\) is the overlap integral, \(n\) the refractive index, and \(\kappa^2\) the orientation factor.
Of perhaps greatest importance in cases where distance variations are of primary interest, is the fact that the $k_0/k_2$ image, i.e. F, is a linear function of $r_{D,A}^{1/4}$, in contradistinction to $E$, and can thus provide reliable relative as well as absolute measures. Relative distance estimations can also be derived in time-resolved measurements, i.e. FRET-FLIM. In three studies based on FRET between an ACP expression probe donor introduced into domain I of EGFR and an acceptor probe localized in the plasma membrane, we applied the following relationship to the lifetime data and thereby confirmed the postulated transition from a close inhibited configuration of the external domain to the open, liganded form of the EGFR dimer (fig. 1).

$$r_{Dm,c} = \left( \frac{r_{Dm,b}}{r_{Dm,a}} \right)^{1/4}$$

in which Dm denotes donor-to-membrane and the subscripts a, b, c the conditions of D alone, D+A, and D+A+EGF, respectively. No parameter other than lifetimes is required in this, admittedly very simplified, calculation. In the cited studies, the second recorded lifetime was FRET relevant and the superscript 1/4 (instead of 1/6) in the equation results from the point-to-plane (single donor to membrane-distributed acceptor) geometry corresponding to the particular experimental design. The expression and others like it are amenable for real-time computation and can thus provide continuous readout in imaging or cytometric assays, including liquid biopsies targeting CTCs, nucleic acids (DNA, RNA), proteins, and peptides.

### A new approach to FLIM (eeFLIM)

The virtues of time-resolved fluorescence lifetime imaging are many and they apply in particular to the determination of FRET. The measurement is relatively insensitive to local concentration, a key factor often limiting the ratiometric techniques in cellular imaging. Both frequency domain and time domain techniques are available in widefield and point scanning systems with high temporal resolution and dynamic range. Such a laboratory instrument incorporating multifocal multiphoton features was applied to the EGFR system. The temporal resolution was excellent (50 ps), and acquisition of a cellular field was achieved in 0.5 s, allowing the visualization of the recruitment of the adapter protein Grb2 to EGFR within 75 s following EGF binding. The inherent optical complexity of this instrument, however, is significant, a property shared with other setups, most of which require the generation of high frequency very short, intense pulses and scanning mechanisms. Alternative frequency domain instruments are generally widefield, an advantage, but feature technical limitations of 2D (camera) phase-sensitive detection. In the case of flow cytometric instruments, lifetime determinations have to overcome the temporal constraints imposed by bulk liquid or cellular movement.

We have addressed the above issues of sensitivity and complexity in FLIM by devising a new, rapid widefield imaging mode, denoted extended excitation FLIM (eeFLIM), and applied it to determine FRET efficiencies in EGFR studies (see below, fig. 5). eeFLIM employs excitation schemes based on extended rectangular light pulses (e.g. 10–60 ns) and records the integrated emission, of a fluorophore or mixture of fluorophores by sweeping the gate of an intensified camera through the synchronized light pulse (fig. 3A). The

### Table 2: Classification of FRET imaging methods. Adapted from ref. 41. The corresponding equations are given in ref. 40. Our lab introduced methods Ia1 (donor photobleaching kinetics), Ie1 (acceptor photobleaching; in widespread use), Ie2 (photochromic acceptor), and le3 (acceptor saturation), and has extensively used la1, lb1, Ia1, Ic1, and Ic2.

<table>
<thead>
<tr>
<th>No.</th>
<th>Method Comments</th>
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<tbody>
<tr>
<td>Ia1</td>
<td>Combined donor (D) and acceptor (A) emission signals</td>
</tr>
<tr>
<td>Ia3</td>
<td>Bioluminescence RET (BRET) New reagents and detection methods</td>
</tr>
<tr>
<td>Ib1</td>
<td>Fluorescence-detected excited state lifetime(s) (FLIM, FLI) Time and frequency domain</td>
</tr>
<tr>
<td>Ib2</td>
<td>Luminescence RET (LRET) New reagents and procedures</td>
</tr>
<tr>
<td>Ib4</td>
<td>Spectral FLIM (sFLIM) Calibration, spectral and temporal resolution</td>
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<tr>
<td>Id1</td>
<td>Donor depletion Improved procedures and analysis techniques</td>
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<tr>
<td>le1</td>
<td>Acceptor depletion FRET (adFRET) Method in widespread use</td>
</tr>
<tr>
<td>le2</td>
<td>Photochromatic A (pcFRET) Reversible adFRET: organic and QD reagents</td>
</tr>
<tr>
<td>le3</td>
<td>A saturation (frustrated FRET) Reversible adFRET: acceptor saturation (new)</td>
</tr>
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</table>

(a) One performs excitations at/near the donor and acceptor absorption peaks (intensities $I_{D,ex}$ and $I_{A,em}$, respectively), and acquires emission signals $(f_{D,em}, f_{A,em})$ in the two corresponding emission bands $(D,A)$. The fixed (constant) ratios in square brackets represent (a) extinction coefficients of the acceptor at the two excitation bands, and (b) relative detection efficiencies of the acceptor or donor at the two emission bands corresponding to the donor and the acceptor. The second and third terms in the expression for $F$ represent direct acceptor excitation and spill-over of donor emission in the acceptor emission band, respectively; inverse overlap can also be incorporated into the equation. Note that the generally invariant (and more experimentally accessible) acceptor quantum yield, $Q_A$, appears instead of the unreliable $Q_D$.
integration process yields much higher signals than those provided by the short pulses required for direct decay analysis. Data evaluation is very simple (involving only non-iterative linear calculations) and is thus rapid. The utilization of all the light emitted per pulse provides a high signal-to-noise ratio (SNR), allowing the acquisition of sequential 1K×1K lifetime images in studies of living cells. The eeFLIM strategy can be easily implemented with laser diodes or LEDs as light sources and with photon counting matrix or single detectors. It is therefore also ideally suited for flow cytometry. The accurate performance of eeFLIM was demonstrated by lifetime determinations of calibration dyes in solution (fig. 3B). Excellent agreement with literature values was achieved.

Another key feature of eeFLIM readily derived from the equations in fig. 3A is that the measurement automatically yields an intensity-weighted mean lifetime for an arbitrarily complex population of fluorophores. This property is demonstrated in fig. 3C with varying mixtures of two dyes in two cases with 10× different relative concentrations. The analysis yields the correct lifetime values including the shortening of rhodamine B due to self-quenching at the higher concentration. The deduced relative intensities (β) reflect the relative values of concentration σ·Q. The automatic calculation of mean parameters is very useful for multiplexed operations such as in drug discovery. This property is not

![Figure 3: Theoretical basis and validation of eeFLIM.](image)

- **A** The emission response function rf[t] using rectangular excitation is an exponential, while its rfi[t] is a linear function of time after attainment of the equilibrium excited state. The key feature of eeFLIM is that the linear responses are displaced on the time axis by values equal to the lifetimes τ. (B) Mean, normalized rfi signals from IRF and four fluorescence dye solutions. IRF: excitation light reflected from the microscope focal plane. The inset highlights the linearity of the signals. The measured lifetimes (table) agree with the literature. (C) Mixtures of nM fluorescein (F) and rhodamine B (R); α = fractional volume F. Solutions of equal concentration (blue) or 10× F/R (green). The global fits (dotted) are to the indicated equation for an intensity-weighted binary mixture. Data of N. Cook, D. Arndt-Jovin, T. Jovin.
shared by methods addressing buildup or decay kinetics directly inasmuch as nonlinear multicomponent exponential analyses are required to identify components and specify the properties and distribution of subpopulations. Scanning and flow cytometry systems based on time-correlated single-photon counting (TCSPC) also fail to provide mean lifetimes “on the fly”. Speed is the issue; eeFLIM requires a minimum of only two time points (total acquisition time of ~0.2 s with the intensified camera) to define the straight-line segments (and thus the lifetimes) shown in fig. 3. With single point detectors acquisition can be arbitrarily short, yielding a continuous real-time stream of mean lifetime values and intensities very well suited for flow cytometers or point scanning imaging systems.

The capability of eeFLIM for registering large contrast in lifetime images is demonstrated by cells stained with the metachromatic dye acridine orange (fig. 4). The very distinct local differences in lifetime reflect the nature of the cellular compartments.

Another eeFLIM experiment addressed the kinetics of ligand (EGF) binding to the EGFR on CHO cells, in this case featuring an Oregon Green label on domain I as donor with TMR-EGF acceptor in the presence or absence of a kinase inhibitor PD 153035 (fig. 5). Both donor and acceptor lifetimes were measured by varying the excitation wavelength. The rapid binding of EGF is reflected in the rapid decrease of donor lifetime and an increase in the acceptor lifetime from the value arising from direct excitation (free ligand) to the higher value reflecting both transfer from the donor (a component with a negative amplitude) and acceptor decay. The lower gradual decrease in donor lifetime can be attributed to the combined effects of conformational rearrangement and membrane redistribution. This experiment demonstrates the value, unfortunately rarely employed, of acceptor as well as donor lifetime determinations in FRET experiments.

eeFLIM was originally devised as a means for incorporating lifetime determinations in a rapid, optical sectioning “intelligent programmable array microscope” (iPAM) under development in our laboratory since 1997⁴⁴. This instrument is unique in the dual use of digital micromirror technology for generating arbitrary patterns of excitation as well as for distributing the evoked fluorescence to two cameras. The recorded conjugate and non-conjugate images are combined so as to effect very high-resolution 3D imaging, including in the 100–200 nm domain of structured illumination microscopy (SIM) and the sub-100 nm domain of certain super-resolution techniques.

Probes for FRET-FLIM
A systematic assessment of probes that fulfill the requirements for FRET and FLIM of cellular targets is beyond the scope of this report; a very useful guide, a comprehensive monograph, has been provided by A. Demchenko⁴⁵. We and others have exploited the unique properties of quantum dots and

Figure 4: Cells stained with the metachromatic dye acridine orange (AO). The fluorescence of AO bound to DNA is green; bound to RNA, green and red; and sequestered in acidic vesicles (lysosomes), red. The lifetime image of the green emission from the nucleus of cell 1 (enlarged) reflects the distribution of DNA and RNA. The RNA-containing nucleoli are particularly distinctive by intensity and (lower) lifetime values. Data of N. Cook, D. Arndt-Jovin, T. Jovin.

Figure 5: eeFLIM of CHO cells expressing EGFR labeled with Oregon Green’ (donor) in the external domain I. The images were taken 1 min after addition of TMR-labeled EGF (acceptor). Both donor and sensitized acceptor lifetimes were measured at the indicated times. Plotted are mean values from the cell surface domains. Data of N. Cook, D. Arndt-Jovin, T. Jovin.
superparamagnetic iron oxide nanoparticles for visualizing and inducing EGFR activation and trafficking\(^{46,48}\). However, one can anticipate targeting molecular properties other than mere presence by use of probing strategies, many based on FRET and FLIM, offering greater sensitivity and selectivity:

- antenna (for a \(kt/kt\) example see ref. 49) and cascade transfer probes;
- signal amplification by numerous strategies such as Tyramide Signal Amplification (TSA) ± bimolecular complementation (e.g. using split horseradish peroxidase) or Proximity Ligation Amplification (PLA);
- cross-linking agents, e.g., multifunctionalized polymers as well as natural or synthetic scaffolds with interaction specificity to induce changes in local density and localization;
- cell-penetrating agents and probes;
- superresolution probes optimized for FRET and FLIM readout;
- expression tags that reversibly bind fluorogenic probes, a useful property for highly multiplexed sequential determinations;
- photoswitchable probes (fluorescent proteins, phytochromes, and photochromic dyes) facilitating FRET in the context of optogenetic or structured illumination (such as in the iPAM\(^{49}\));
- refined expression probes providing for exchangeable fluorogenic ligands, ideal for multiplexing in the spatial, temporal, and functional dimensions; and
- probes optimized for Single Particle Tracking (SPT) and Fluorescence Correlation Spectroscopy (FCS).

In many applications, donors with large Stokes shifts are very advantageous or even required, as are defined polarization states enabling anisotropy determinations. In addition, other light emission modalities such as delayed fluorescence and phosphorescence deserve renewed attention, particularly in critical time-resolved measurements requiring efficient background suppression. Finally, one can anticipate the exploitation of a recently introduced, radically expanded 8-base genetic code with corresponding microorganisms able to process such “Hajimochi” DNA and RNA\(^{31}\). The far-reaching implications of this achievement cannot be overstated. At the very least, innumerable expression probes with novel properties should become available.

**Concluding remark**

We would like to express our appreciation to Christian Dose for inviting us to contribute to this special issue of MACS&more commemorating the 30th anniversary of the founding of Miltenyi Biotec and the pioneering, inspiring leadership of Stefan Miltenyi ever since! Congratulations to him and to his colleagues!

**References**

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Development of cellular immunotherapies for solid cancers of childhood

Pediatric oncology faces particular challenges
Pediatric oncology is a prominent example of the efficacy of multimodal combination therapies against malignant diseases, and of the value of randomized clinical trials with national and international cooperation to systematically improve the outcome (fig. 1). Nevertheless, it has become apparent that further increases of the probability of relapse-free survival in pediatric cancers requires additional treatment modalities that act by fundamentally different mechanisms than standard cytotoxic agents. The striking activity of allogeneic immune responses in the context of hematopoietic stem cell transplantation in some leukemias has unequivocally demonstrated the potential of cellular therapy to induce remissions in chemoresistant cancers, and to create plateaus of long-term remissions. In solid tumors, however, allogeneic transplant has not shown convincing activity. More recently, the remarkable efficacy of immune checkpoint inhibitors to induce and maintain therapeutic antitumor immune responses in the microenvironment of melanoma and some carcinomas has renewed the interest in a role of the cellular immune system in fighting solid cancers. However, in classical solid tumors of childhood, checkpoint inhibitors are not effective, for the following reasons: The types of cancer occurring at pediatric age lack infiltrating T cells as well as PD-L1 expression, and are characterized by a very low tumor mutational burden. Thus, these cancers lack the critical prerequisites determining the action of checkpoint inhibitors. A typical pediatric tumor represents an "immunological desert" and is ignored by the cellular immune system. For effective immune targeting in the absence of infiltrating immune effector cells and neoantigens recognizable as foreign, therapeutic antigen-specific T cells will have to be actively introduced into these tumors.

Figure 1: Two-year overall cancer survival rates of children in Germany from 1940 to 2010. Adapted from ref. 1.
T cell engagers and engineered T cells – potential therapeutic tools in pediatric oncology

An off-the-shelf option to redirect T cells to tumor-associated antigens are bispecific T cell engagers. The best example today is blinatumomab, which is effective to induce remissions in a proportion of both pediatric and adult leukemia patients⁷, but these responses often do not last and can only bridge to subsequent allogeneic stem cell transplantation. Bispecific T cell engagers are under development for several other cancers, including GD2 in neuroblastoma, but whether these small molecules can effectively redirect circulating T cells to a solid tumor remains to be shown.

An alternative are chimeric-antigen receptor (CAR)–modified T cells which are generated by gene transfer⁸ (fig. 2A). CARs are synthetic receptors which link antigen binding domains to the T cell receptor ζ chain and to costimulatory signals, to redirect T cell activation responses to tumor-associated surface antigens. CAR T cells directed against the B lineage antigen CD19, combined with lymphodepleting chemotherapy, were found to induce complete responses in chemorefractory CD19-expressing leukemias and lymphomas, with remarkable plateaus of event-free and overall survival after almost two years without subsequent transplant⁹. Phase II studies have led to approval of the first CD19 CAR T cell products in 2018. In solid tumors, CAR T cells have had limited activity so far¹⁰–¹².

Another way to create tumor antigen–specific T cells is to engineer autologous T cells to express affinity-enhanced TCRs against cancer testis antigens (fig. 2B), such as NY-ESO-1, which is strongly and diffusely expressed in 70% to 80% of synovial sarcomas. Adoptive transfer of the gene-modified T cells in 12 patients with advanced synovial sarcomas led to six confirmed responses, proving the principle that adoptive T cell therapy can be effective even in sarcomas¹³. For a substantial impact in childhood solid tumors, these strategies clearly need additional advances. Combination partners or designs are needed that recruit anti-tumor effector T cells to the tumors, prevent their functional inactivation in the hostile microenvironment, and overcome the inadequate, heterogeneous expression of most known tumor-associated antigens.

Strategies to enhance T cell–based therapy

There is indeed evidence that CAR T cell therapy can raise adaptive immune resistance: In a clinical study of EGFRvIII–specific CAR T cells in patients with refractory gliomas, post-therapeutic biopsies demonstrated strong up-regulation of PD-L1 in tumor tissue¹². Thus, an obvious next step in CAR T cell therapy of solid tumors is to combine it with checkpoint inhibitors against PD-L1/PD-1 and/or other receptors that contribute to inactivation of CAR T cells in the tumor microenvironment. The same clinical study revealed an additional problem that cannot be overcome alone by combining CAR T cell transfer with checkpoint inhibition: In most patients receiving CAR T cells, expression of the target antigen within the tumor declined or even disappeared; disease in most patients progressed and the patients died¹³. Cotargeting more than a single antigen is a reasonable approach to preventing escape. In addition, agents may be identified that selectively up-regulate cancer-associated antigens. Candidates are epigenetic agents which have raised much interest especially in the context of pediatric tumors where mechanisms of epigenetic gene regulation have important roles in tumor pathogenesis. Such agents are also able to induce expression of NY-ESO-1 and other cancer testis antigens, to allow recognition by antigen-specific T cells. A proof-of-concept study showed that decitabine combined with doxorubicin enhances expression of the NY-ESO-1 antigen by promoter hypomethylation, resulting in in vitro T cell recognition¹⁴. Five of 11 patients treated with this combination had increases of either pre-existing or undetectable NY-ESO-1–specific CD8+ T cell responses. This could be a potent strategy to be combined with NY-ESO-1–specific T cell transfer.

Epigenetic agents have multiple functions on cancer cells, immune cells, and the

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Figures 2A and 2B: Schematic of T cells recognizing tumor-associated surface antigens through CARs (A) or tumor neoantigens/cancer testis antigens through engineered, affinity-enhanced TCRs (B). Notably, the initiation of CAR T cell activity does not require antigen presentation by MHC molecules.
tumor microenvironment and therefore are attractive agents to sensitize cancer cells to immunotherapy. However, any of their effects could also be counterproductive, and we will have to understand their impact on all the relevant cell populations involved in a productive immune response in detail to choose the right combination partners.

Overall, additional target antigens must be identified that are selectively expressed on the surface of all cells of the tumor but not on normal cells. Alternatively, or in addition, intelligent targeting strategies are needed that overcome heterogeneous antigen expression or cross-reactivity of engineered T cells with normal cells, e.g., by Boolean logic designs.

**Tumor microenvironment – a critical factor in cellular therapy**

The nature of the immunosuppressive microenvironment in childhood cancers is only beginning to be investigated. While often devoid of effector T cells, it contains remarkably high proportions of CD68- and CD163-co-expressing macrophages and other myeloid cells. Such cells can have immunosuppressive function and may be serious obstacles to T cell recruitment and optimal function even in adoptive T cell therapy. Novel analytic tools now allow us to understand complex cellular environments by single-cell gene expression analysis and by phenotyping cells within their local microenvironment in direct relation to their neighbors. In a recent study including 33 cancers occurring in adults, individual immune gene and protein signatures as well as signaling networks were analyzed along with quantification of immune cell subpopulations. Six different immune subtypes were identified, from highly inflammatory wound healing phenotypes to lymphocyte-depleted and entirely quiet types. Not surprisingly, the few entities overlapping with pediatric oncology were mostly lymphocyte-depleted and immunologically quiet. Another question not yet addressed in childhood tumors is the molecular basis for their lymphocyte-depleted microenvironment. There is increasing evidence that tumors shape their own environment, as shown in a recent study where single cell clones of pancreatic tumors from areas containing high or low proportions of T cells were grafted into mice. The tumor clones created very different immune microenvironments either responding or not responding to immunotherapy. Similar analyses in pediatric cancers could help to identify critical obstacles for cellular therapies and thereby inform the design of optimized strategies.

**Technical and organizational challenges in the development of cellular therapies**

A challenge to the academic community in the clinical development of adoptive cellular targeting strategies is the requirement for individualized T cell manufacturing by pharmaceutical quality standards which, compared to off-the-shelf agents, is complex and expensive. Clearly, cellular therapy is unlikely to be active on its own. To establish the value of T cell therapy in comparison with the current gold standards and to define the optimal combinations and position of cellular immunotherapies in existing treatment algorithms, advanced-phase clinical multicenter trials initiated and performed by the well-established international pediatric oncology study groups are needed. Such trials require close communication and cooperation between academic investigators and industrial partners. Specifically, technical and logistic solutions must be found that allow the manufacture and distribution of highly equivalent engineered T cell products at sufficient scale and affordable cost. Uniform GMP standard operation procedures, reliable supplies of GMP reagents, automation and simplification of the manufacturing process are needed to ensure quality and minimize risks, labor intensity, and cost. Tools are also needed to maximize the gain of knowledge from clinical trials by harmonized immune monitoring programs. Concerted action also with regulatory authorities may facilitate the advancement of cellular therapy as a future pillar of cancer therapy, both in adult and pediatric oncology.

**References**

25 years of development: dendritic cells for anti-tumor immunity

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Dendritic cells (DCs) are potent antigen-presenting cells and thus have the capacity to induce anti-tumor immunity. DCs naturally occurring in the blood can be manufactured fast and in a highly standardized and automated fashion. This can improve the quality of DC vaccines and enables multicenter clinical trials.

Dendritic cells – sentinels of the immune system
Dendritic cells (DCs) were discovered in 1973 by Cohn and Steinman1. DCs play an important role in the induction of immunity: Spread throughout the body, they are the sentinels of the immune system that monitor their surroundings for antigens and danger signals derived from pathogens or tissue damage. They are the most potent antigen-presenting cells, able to initiate and modulate specific immune responses. Following infection or inflammation they undergo a complex process of maturation and migrate to lymph nodes where they present antigens to T cells. Their decisive role in inducing immunity formed the rationale for DC-based immunotherapy: DCs loaded with tumor antigens are injected into cancer patients to stimulate T cells that recognize and eliminate cancer cells and induce immunological memory to control tumor growth2.

Blood-derived dendritic cells as potential tumor vaccines
While worldwide mainly monocyte-derived DCs (Mo-DCs) are used in clinical vaccination trials, it is questionable whether these cells are the optimal source of DCs for the induction of potent immune responses. The extensive culture period (8–9 days) and compounds required to differentiate monocytes into DCs may negatively affect DC migration and costimulation, perhaps by exhaustion3–9. This prompted us to consider alternative DC sources for vaccination, e.g., DCs that are naturally circulating in the blood. Two major types of naturally occurring DCs are present in the blood10–13, which can be distinguished by the presence of different surface markers: plasmacytoid DCs (pDCs) and myeloid DCs (mDCs).

1) pDCs, which are thought to be derived from lymphoid precursors and have plasma-cell like morphology, produce large amounts of type I interferons in response to viral or bacterial stimuli14–16. Human pDCs express CD123, CD303 (BDCA-2), and CD304 (BDCA-4), but lack the myeloid antigen CD11c17.

2) mDCs are characterized by the presence of CD11c and CD1c and have been linked to T helper 1 (Th1) cell induction18. mDCs and pDCs express a different repertoire of pattern recognition receptors and we demonstrated that they respond differentially to extracellular stimuli19,20. In addition, the DC subsets exhibit different migration patterns21,22. mDCs reside in almost every organ or tissue16,23. They are considered to exhibit the following in vivo trafficking pattern: Following antigen uptake in peripheral tissues, mDCs exit these tissues in an immature state and migrate via afferent lymphatics to T cell areas of regional lymphoid tissue, where they – in an activated state – induce T cell activation and proliferation. By contrast, pDCs in the blood follow a different path: During inflammation, immature pDCs appear to exit the blood via high endothelial venules for direct entrance into draining lymph nodes24,25. These different expression patterns of pathogen recognition receptors and migratory behavior of mDCs and pDCs suggest that they may support different yet complementary functions in the lymph node11,26,27. Blood-circulating mDCs and pDCs constitute only about 1% of blood mononuclear cells. Novel isolation techniques recently made it feasible to isolate these DCs directly from blood of patients. Because the isolation is performed by a fully closed standardized method, based on the CliniMACS Prodigy®, the execution of multicenter trials is within reach.

Clinical trials employing DC subsets to induce anti-tumor immunity
To date, seven clinical trials with blood-derived DCs are performed in cancer patients, all using the CliniMACS® System from Miltenyi Biotec for immunomagnetic
Isolation and maturation of mDC vaccines

To isolate mDCs, first CD19+ B cells are depleted and then CD1c+ cells are positively selected by the respective CliniMACS® Reagents. In our clinical trials in melanoma and prostate cancer patients, this procedure results in mDC purities of around 90% and yields between 30×10⁶ and 100×10⁶ cells starting from 12 L of apheresis material. In these trials, the isolated cells are stimulated with GM-CSF, resulting in semi-mature mDCs that are HLA-ABC/DQ/DR+, CD86+, and show variable CD83 and CD80 expression. We recently observed that CD1c+ mDCs also expressing the monocytic marker CD14 suppress CD4+ T cells and their immune suppressive effect may severely hamper DC vaccination efficacy. Therefore, we now deplete this cell population from the mDC product. To further enhance the capacity of the mDCs to stimulate antigen-specific T cells, protamine/mRNA complexes were identified as a maturation factor since they bind to TLR8 on mDCs. Moreover, these complexes can also stimulate pDCs via TLR7. Upon protamine/mRNA stimulation, mDCs produce the Th1 cell-polarizing cytokine IL-12p70, whereas pDCs produce IFN-α. On both subsets, enhanced expression of HLA class I, CD80, and CD86 was observed after protamine/mRNA stimulation. Consequently, the stimulated DCs were able to induce T cell proliferation and activation. The capacity of protamine/mRNA to activate both mDCs and pDCs makes it possible to combine the two subsets in one DC vaccine (see below).

Isolation and maturation of pDC vaccines

The pDCs are isolated by the CliniMACS CD304 (BDCA-4) Reagent, without any prior depletion step. The purity of the pDCs is slightly lower (average purity of 75%) compared to mDCs. As expected, based on the number of pDCs in peripheral blood, the yield is also lower compared to mDCs, ranging from 13×10⁶ to 33×10⁶ cells. In our previous trial with pDCs, maturation was induced with Frühsommer-meningoencephalitis (FSME; also termed tick-borne encephalitis) vaccine. Stimulation of pDCs with the FSME vaccine via TLR7 resulted in both cytokine production and maturation, making it a suitable maturation agent for pDCs. As described above, protamine/mRNA is also able to enhance the expression of maturation markers and induce cytokine production (IFN-α) by pDCs.

Combined mDC and pDC vaccines

The co-application of pDCs and mDCs might have a synergistic effect on the immunostimulatory capacity of blood-derived DCs, due to the complementary features of pDCs (production of large amounts of type I IFN) and mDCs (production of IL-12p70). In vitro, a synergistic effect of DC subsets on NK cell function could be confirmed. Additionally, we studied the crosstalk between mDCs and pDCs regarding their release of IL-12p70 and type I IFN, respectively. The large release of type I IFNs by pDCs led to a more potent activation of mDCs upon coculturing of the two subsets. Besides the increased activating effect on innate immune cells, like NK and NKT cells, an increased production of IFN-γ by T cells upon coculture with the combined DC subsets was observed. These findings led to the development of a combined vaccine composed of pDCs and mDCs isolated, cultured, and stimulated (protamine/mRNA) together (fig. 1). We hypothesize that these...
combined DC subsets have a strong potential to induce immune activation and thereby increase the efficacy of the immune response in vivo. A large randomized trial performed with this combined DC subset vaccine is awaiting results (see below).

Toxicity effects of DC vaccination

Of all completed trials with blood-derived DCs the primary endpoint was safety and the secondary endpoint was an immunological endpoint. DC vaccination with Mo-DCs has shown to cause minimal toxicity as demonstrated in numerous small trials and several larger trials. The few side-effects that are known for DC vaccination mainly consist of flu-like symptoms, including fever, and injection site reactions. Grade 3 or 4 adverse events are extremely rare. For DC subset vaccination the data thus far is limited, but the toxicity seems to be similar or even more favorable than for Mo-DCs.

Survival

To date, little can be concluded on the clinical efficacy of blood-derived DC vaccination. In our trials with advanced cancer patients, progression-free survival (PFS) was short in most patients. However, in the trial with mDCs the PFS was longer in patients with tumor-specific T cells compared to patients without tumor-specific T cell. Furthermore, despite the short PFS in most patients, overall survival seemed to be relatively long. However, overall survival might be biased by subsequent treatments, such as immune checkpoint inhibitors. More robust survival data should be obtained from the first phase III trial with DC subsets. We started this trial in 2016 and employed the combination of pDCs and mDCs, and this treatment is compared to placebo in the adjuvant setting in stage III melanoma patients.

Conclusion

Although DC subsets are very scarce in peripheral blood, sufficient cells for DC vaccination can be obtained by immunomagnetic isolation. The advantage of DC subsets lies in the rapid and highly standardized, automated production of the vaccines, which can improve the quality of the DC vaccines and enables multicenter trials. Only limited data from completed clinical trials with DC subsets are available, which however show promising results with very limited toxicity, even in comparison to Mo-DC studies. Subsequent trials as well as data from ongoing trials will have to substantiate the role of DC subsets in DC-based immunotherapy.
Adapter CAR™ Technology – the missing link towards controllable CAR T cell function

**CAR T cell–based therapy fighting liquid and solid tumors**

CAR T cell therapy focusing on the elimination of CD19+ neoplastic B cells has resulted in high remission rates in blood cancers such as acute lymphoblastic leukemia (ALL) and non-Hodgkin’s lymphomas (NHL). This ultimately resulted in the approval of two therapies1,2 by both the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Myriads of clinical trials using gene-modified T cells are being pursued in order to explore new ways to fight different kinds of blood cancer as well as various types of solid tumors3,⁴. Treating solid tumors by CAR T cells however is more challenging than tackling the “liquid” tumors, because it is even harder to define antigens that are really tumor specific. In addition, it is more difficult for CAR T cells to reach the target cells as they first need to infiltrate the solid tissue where they face a hostile environment including suppressive immune cells⁵–⁷.

**Intrinsic CAR T cell functions can compromise therapy**

CAR T cells are designed to kill the cells that express a defined tumor-associated antigen (TAA). In many cases though, this antigen is not only expressed by the tumor cells but also by healthy cells. This results in on-target/off-tumor toxicity. CD19 CAR T cells, for example, will not only kill neoplastic CD19+ B cells but also healthy B cells that produce antibodies protecting against infections.⁵ In this case, the eliminated healthy B cells can be substituted by immunoglobulin replacement.⁵ However, when the TAA is expressed on essential tissues, destruction of these tissues can be life threatening.

Moreover, as soon as CAR T cells have encountered their antigen, they start to proliferate and release cytokines, which in turn can lead to the so-called cytokine release syndrome (CRS).⁶–⁸ Although CRS is a potentially life-threatening condition, it can be handled well by immunosuppression with tocilizumab, an FDA-approved drug for severe CRS, which competes with IL-6 for binding to the IL-6 receptor.⁹

Furthermore, CAR T cells directed against a single target antigen exert a high selection pressure on the tumor, which can lead to down-regulation or loss of the targeted antigen on the malignant cells and thus might result in the so-called antigen escape10,11. In other words, CAR T cells fail to recognize and kill the tumor cells, which ultimately can cause a relapse of the disease.

A number of concepts aim to avoid all these drawbacks through mechanisms that enable the control of CAR T cell function as well as the targeting of multiple antigens.

**CAR T cells require control**

Various strategies have been developed to control detrimental effects of CAR T cell therapy.⁶,¹³ These approaches include for instance i) pharmacological immunosuppression, ii) genetic modification of CAR T cells to include so-called “suicide genes” that would enable the abrogation of excessive CAR T cell function, iii) use of CAR T cells that only become fully active when different defined antigens are present on the target cells, iv) a strategy to avoid continuous activation of CAR T cells by using an adapter molecule that links
the CAR to the TAA on the tumor cell. All these strategies allow for a control of CAR T cell function, albeit with varying specificity and reversibility.

**Adapter CAR™ Technology – a novel approach pursued by Miltenyi Biotec in collaboration with partners**

CAR T cells that become effective on target cells indirectly through adapter molecules represent one of the most promising approaches towards controllable CAR T cell therapy. Researchers from Miltenyi Biotec in collaboration with partners from the University of Tübingen, Germany set out to explore the potential of this novel strategy with two main objectives in mind: It should be possible to turn CAR T cells on and off “at will”, and there should be the option to target multiple antigens in parallel or consecutively. These features could solve numerous challenges associated with CAR T cell therapies.

**Molecular principle underlying Adapter CAR™ Technology**

Binding of Adapter CAR™ T cells to their target, i.e., cancer cells, occurs indirectly through a defined adapter molecule. This adapter molecule consists of a tag that is recognized specifically by the CAR and a portion that binds specifically to an antigen on the target cells (fig. 1). The part of the adapter molecule that binds to the target cell can consist of, for example, an antigen-binding fragment (Fab) of a TAA-specific antibody. This configuration allows for an on/off switch as the CAR T cells bind to the target cells (and thus become activated) only in the presence of the adapter molecule, which can be dosed transiently (fig. 1).

**Proof-of-concept experiment holds great promise**

In proof-of-concept studies, we tested the effectiveness of Adapter CAR T cells in a mouse model of lymphoma. The adapter molecule consisted essentially of i) a Fab that binds to a TAA expressed by the target cells and ii) a specific tag moiety for binding to the Adapter CAR T cells (fig. 1). NSG mice were injected with luciferase-expressing Burkitt’s lymphoma cells leading to tumor growth. After five days, standard CAR T cells, which bind to the TAA directly, or Adapter CAR T cells with or without the anti-TAA adapter molecule were administered (fig. 2A). Luciferase activity was monitored over time to assess the cytotoxic potential of the CAR T cells. While injection of standard CAR T cells led to a direct reduction of luciferase activity, Adapter CAR T cells were only active in the presence of the anti-TAA adapter molecule. Adapter molecule in combination with mock (i.e. non–CAR-expressing) T cells did not exhibit robust antitumor effects (fig. 2B). Further detailed studies are ongoing (manuscript in preparation).

Among the many strategies that have been designed to refine CAR T cell–based therapy, the combination of Adapter CAR T cells and corresponding adapter molecules is one of the most promising approaches as it offers many advantages over standard CAR T cells.

Taken together, we expect this concept to combine the strong capacity of CAR T cells to eliminate tumor cells with the precise pharmacological control exerted in therapies using “traditional drugs”.

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**Figure 1: Mechanism of Adapter CAR T cells.** Standard CAR T cells are always turned on as their single-chain variable fragment (scFv) binds directly to the TAA expressed on the surface of the target cell. In contrast, Adapter CAR T cells only become active in the presence of the adapter molecule, which can be added or withdrawn as desired and thus provides an on/off switch mechanism. Importantly, Adapter CAR T cell action can be fine-tuned i) by precisely adjusting the serum concentration of the adapter and thereby controlling the T cell response, ii) by using different target cell–binding portions for the adapter molecule, iii) by applying different adapters that bind to distinct antigens on the target cells.
Adapter CAR™ Technology offers numerous benefits:

- A single, universally usable type of CAR T cell can be applied to address various target antigens because the specificity of binding to the target cells is defined by the respective adapters. In this context, it is important to note that dosing of adapter molecules is highly controllable, whereas direct CAR T cells can expand and react very differently in different patients.

- Due to the possibility of addressing multiple antigens, target cells can be attacked in various ways, simultaneously or consecutively. This feature is particularly important in the context of solid tumors where cancer cells are highly heterogeneous, expressing different sets of TAA on their surface.

- The option to tackle multiple antigens also helps to circumvent a potential antigen escape.

- Fine-tuning of the anti-tumor response is made possible by selectively adding or omitting adapter molecules with different specificities, thus providing a solution for personalized medicine.

- The effect of CAR T cells depends on the concentration of the adapter molecule, which not only enables on/off switching, but also gradual fine-tuning of the anti-tumor response.

- The possibility of fine-tuning minimizes on-target/off-tumor effects, and thus should reduce potential side effects of future CAR T cell–mediated therapies.

- Adapter CAR Technology is fully compatible with approaches to overcome the hostile tumor microenvironment in solid tumors. This includes immune checkpoint inhibition, enhancement of T cell migration into the tumor by expression of chemokine receptors on the T cell surface, and increased expression of cytokines (e.g. IL-12, IL-15, IL-18, or IL-21) by CAR T cells to modulate the tumor microenvironment.

**Figure 2: Proof-of-concept experiment for Adapter CAR T cells, based on a mouse model of lymphoma.**

The experiment was performed with standard anti-TAA CAR T cells or with Adapter CAR T cells +/- anti-TAA adapter. The adapter molecule consisted of an anti-TAA Fab fragment and a tag moiety for specific binding to the Adapter CAR T cell. Luciferase-expressing Burkitt’s lymphoma (Raji) cells (3×10⁵ cells) were injected into NSG mice, resulting in tumor growth. After 5 days (day 0), mice were treated with standard CAR T cells without adapter, Adapter CAR T cells with adapter, Adapter CAR T cells without adapter, or normal T cells with adapter (1×10⁷ cells each). From day –1 to day 18, the adapter molecule was given i.p. daily (A). Luciferase activity was monitored over time using an in vivo imaging system to assess the killing potential of the T cells (B). Experiment was performed by Fabian Engert, Britta Drees, and Sandra Dapa. Adapter molecules were generated by Jonathan Fauerbach and Jörg Mittelstädt.

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Production of GMP-grade gut-homing regulatory T cells at the NIHR Biomedical Research Centre, Guy’s and St Thomas’ Hospital and King’s College, London, UK

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Crohn’s disease causes inflammation of the gastrointestinal tract. To date, no curative therapy is available. However, recent findings suggest that a lack of gut-homing regulatory T cells is a cause of the disease, providing the basis for clinical trials towards novel cellular therapy.

Inflammatory bowel disease (IBD) is a progressive immune-mediated disease which affects a significant number of people. In the UK alone, there are ~300,000 people with either Crohn’s disease or ulcerative colitis1. Further to this, the incidence of IBD exceeds 0.3% when the cumulative population of Canada, Denmark, Germany, Hungary, Australia, New Zealand, Sweden, the UK, and the USA is assessed2. The distinction between the two main IBD conditions (ulcerative colitis and Crohn’s disease) is macroscopically in the affected anatomical region. Ulcerative colitis is restricted to the large intestine, whereas Crohn’s disease can manifest throughout the entire length of the gastrointestinal tract. The symptoms of these conditions vary between individuals, however the damage caused by aberrant inflammation is progressive and can lead to significant digestive problems like malnutrition, bleeding, and intestinal obstruction. Current therapies for these conditions focus on interrupting or dampening the inflammatory process with immunosuppressants or biologics, but no lifelong cure exists. Approximately 1/3 of Crohn’s disease sufferers will require surgery, to remove damaged tissue or correct obstruction, however this comes with long-term health implications and a large economic burden for the health service. Novel approaches to treating these diseases are therefore required.

The maintenance of inflammatory homeostasis in the gut relies on a delicate balance between pro-inflammatory T cells preventing pathogens from invading the body and regulatory T cells reducing aberrant inflammation. A recent study in our research lab at King’s College London has identified an imbalance in regulatory T cell homing between healthy individuals and Crohn’s disease patients. The healthy individuals had a significantly greater frequency of gut-homing (integrin α4β7) regulatory T cells circulating in their peripheral blood, than the Crohn’s disease patients. This finding was also observed in biopsy samples from the large intestinal mucosa. This regulatory T cell homing deficiency could explain the inflammatory imbalance driving the symptoms of Crohn’s disease4. To put it in other terms, if this deficiency could be corrected, would the disease resolve?
We are aiming to answer this very question and will start trials of a gut-homing regulatory T cell product in mid 2019 (clinicaltrials.gov no: NCT03185000). A novel molecule has been tested by our research lab that can induce the stable expression of integrin α4β7 on the surface of polyclonal regulatory T cells. Using knowledge recently developed in our laboratory of ex vivo polyclonal regulatory T cell expansion and manufacture⁴, and utilizing Miltenyi Biotec’s cell separation and sorting technology available in the Biomedical Research Centre Advanced Therapies manufacturing facility at Guy’s Hospital, the production of clinical-grade gut-homing regulatory T cells is now possible. An illustration of a patient’s journey through this novel trial is shown in figure 1.

**Figure 1:** Timeline of Crohn's disease patient enrolled into the Tribute trial.

References


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MACS® Technology, mutants, and microbes

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Originally developed in the context of the enrichment of mutant hybridoma cells, MACS® Technology soon became a standard method for the isolation of virtually any cell type. Deciphering intestinal microbiota is one of the most recent research applications, ultimately leading to a better understanding of host-microbiota interaction in health and disease.

The quest for antibody-secreting cell lines
The Institute for Genetics of the University of Cologne was established in 1962 by Nobel laureate Max Delbrück. It soon became a center of research in molecular biology with landmark discoveries on gene regulation, the epigenetic imprinting of genes, their mutation and targeted mutagenesis. In the Department of Immunology, headed by Klaus Rajewsky since 1970, genetic methods were developed and used for the analysis of the immune system, in particular B lymphocytes and the antibodies they produce. One of the key approaches was the isolation of genetic mutants and their comparison to wild type cells. Luckily, in the year 1975, the department could acquire the fourth fluorescence-activated cell sorter ever built by the company Becton-Dickinson, the first such machine in Germany. At the same time, in Cambridge (UK), Köhler and Milstein originally described their method to immortalize activated antibody-secreting B lymphocytes, or plasmablasts. This technology was rapidly transferred to Cologne and used to generate a plethora of “monoclonal” antibody-secreting cell lines, the hybridomas.

Challenges in the isolation of mutant hybridoma cells
In 1976, Andreas Radbruch joined the department to do a cytogenetic analysis of hybridoma cells for his diploma thesis. For his Ph.D., he then joined the team working with the fluorescence-activated cell sorter. Together with Bernhard Liesegang, he developed a method to isolate spontaneous mutant hybridoma cells secreting antibodies of a class or specificity different from the original hybridoma. Such cells were rare, occurring at frequencies of $10^{-6}$ to $10^{-5}$, making their isolation extremely tedious and difficult. Isolation of such mutants lasted for hours, sometimes even overnight while trying to maintain sterile conditions for subsequent cultivation. Isolation of mutants usually involved repeated rounds of enrichment by fluorescence-activated cell sorting. The isolation of a particular mutant took weeks, if not months at a time. Despite these difficulties, the project line turned into a success and a series of publications on antibody class-switch recombination, trans-chromosomal recombination, gene conversion, and idiotypic point mutations and their effect on antibody specificity and function was the result. The team was expanded as Marianne Brüggemann, Bodo Holtkamp, Christa Müller-Sieburg, Michael Neuberger, John Kearney, and others joined the group, and the isolation of hybridoma mutants by fluorescence-activated cell sorting became a hallmark of the Rajewsky lab in the 1990s. From the start, the team was exploiting options to enrich the mutants prior to fluorescence-based sorting, by “panning”, killing of wild type cells, “sequential sub-lining”, upgrading of the cell sorting process – with limited success. Isolation of the mutants remained inefficient.

MACS® Technology – A success story opens up new horizons for cell research
In 1988 – meanwhile Andreas Radbruch had become head of the cell sorting facility – a new student joined his group. Stefan Miltenyi had studied physics and was interested in its biomedical application. His vision was to develop a fully automated lab, a vision which we could not pursue at that time, simply because of lack of resources, but which today is turning into reality at Miltenyi Biotec. In the cell sorting group, Stefan Miltenyi began to exploit magnetic cell sorting approaches to pre-enrich cells of interest for subsequent fluorescence-activated cell sorting. Not to interfere with fluorescence-based cell sorting, the magnetic label had to be very small. Therefore, we decided to focus on labeling cells with superparamagnetic nanoparticles and enriching them in high-gradient magnetic fields, generated in columns filled with steel wool in an external magnetic field. Stefan Miltenyi introduced two decisive features to this magnetic cell separation technology,
A new application, which is currently worked on at Miltenyi Biotec, is the identification of microbiota. Analysis of intestinal microbiota – Gold standard for determining microbiota composition is high-throughput sequencing of the 16S rDNA, while isolation of specific bacteria is done by classical microbiological cultivation. Both techniques have their limitation: 16S rDNA sequencing is prone to bias and methodological variation, whereas microbiological cultivation is limited to those species whose growth conditions are known, or requires substantial resources and know-how. The application of MACS® Technology and fluorescence-activated cell sorting in microbiota analysis offers the potential to resolve the composition of a bacterial community on the single-cell level and to isolate specific bacteria directly. Together with the group of Susann Müller from the Helmholtz Centre for Environmental Research in Leipzig, Germany, we have developed high-resolution microbiota cytometry for the analysis of intestinal microbiota. By quantitatively staining the DNA of bacteria combined with sensitive light scatter detection, we can resolve in the order of 20–70 bacterial communities. This already allows us to monitor dynamic changes in the microbiota composition over time, e.g., during development of disease. The group of Andrey Kruglov at the DRFZ is developing monoclonal antibodies directed against specific bacteria which will allow us to combine DNA content and scatter analysis with specific staining and the development of truly multidimensional microbiota cytometry. Already now, staining of intestinal microbiota with anti-isotype antibodies, in particular anti-IgA antibodies or IgG antibodies in cases where the intestinal epithelial barrier has been disrupted, identifies bacteria which have potentially come into contact with the immune system and are of particular interest in immune-mediated diseases. Isolating specifically labeled bacteria can be even more challenging than sorting rare hybridoma mutants, as the number of bacteria is very large. One gram of stool can contain more than 10¹³ bacteria comprising several hundred species. Thus, when processing bacteria one has to deal with cell numbers and complexity several orders of magnitude higher than in experiments with eukaryotic cells. This is where MACS Technology, which allows parallel cell isolation, can again play out its strength for the enrichment of rare bacteria, in combination with fluorescence-activated cell sorting. The compactness of the MACS Cell Separation system also allows for the sorting of bacteria under oxygen-free conditions in anaerobic cabinets giving access to oxygen-sensitive bacteria for downstream analyses.

References

Analysis of intestinal microbiota – facilitated by MACS® Technology
A new application, which is currently worked out in the group of Hyun-Dong Chang, is the cytometric analysis of complex bacterial communities, such as the intestinal microbiota. Gold standard for determining microbiota composition is high-throughput sequencing of the 16S rDNA, while isolation of specific bacteria is done by classical microbiological cultivation. Both techniques have their
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Make cancer history.
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The cover image shows a snake embryo after tissue clearing and staining for β-III tubulin (axonal marker, cyan) and FoxP2 (neuronal marker, magenta). The image was generated by light sheet microscopy (see article on p. 10).

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