CoMMiTMenT

Combined Molecular Microscopy for Therapy and Personalized Medication in Rare Anaemias Treatments

Final Report

4.1 Publishable Summary

Project Duration:	01/10/2013 to 30/09/2018
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1 Executive Summary

The CoMMiTMenT project developed a set of novel tools for the detection, selection and complex characterisation of the vulnerable sub-population of red blood cells in the blood of patients with rare anaemia. Those tools identified as being appropriate to combine have been combined. Novel devices have been successfully fabricated by the SMEs in the consortium and tested by the academic and clinical partners. These devices make use of cellular properties like their labelling with fluorescent molecular biomarkers (e.g., antibodies or fluorophores), secondary cellular characteristics, such as the cell shape, or other alterations of cellular morphology and structure as well as cellular interaction with probing media and surface structures. The technology partners brought to the project expertise in micro and nanotechnology particularly microfluidics, optoelectronics and imaging software that were combined into systems (the µCOSMOS concept) for red blood cell testing. By utilising a smart combination of the above mentioned techniques two marketable diagnostic instrumentation products have been prototyped and tested in both research and hospital clinical environments (achieving TRL6). The first is a Scanning Ion Conductance Microscope (SICM) marketed by OpenIO Labs with demonstrated benefits in examining, for example, Gardos channels. The second developed by Epigem Limited combines microfluidics and optoelectronics and examines the morphology of red blood cells and utilises novel test procedures proposed and tested by UZH using mechanical and chemical probes. This has become known as “MeCheM”, combining in the name the Mechanical and CheMical procedures employed and delivers the µCOSMOS concept. Two patent applications have been filed. MeCheM is already building into a portfolio of test methods that combine multiple analytical test methods to simplify, accelerate, reduce results uncertainty, and increase diagnosis utility e.g. mutation identification. A MeCheM prototype is already being successfully used for monitoring therapeutic interventions in rare anaemias for the treatment of sickle cell disease. Hereditary xerocytosis, overhydrated hereditary stomatocytosis, familial pseudohyperkalemia, cryohydrocytosis, certain types of spherocytosis, hereditary spherocytosis, sickle cell anaemia, thalassemia and phosphofructokinase deficiency have been tested and reached different exploitation levels. The non-invasive nature of the combined imaging technologies enables to probe the function of molecular effectors and facilitates the testing of medications and their dosage in a personalised manner. For some of the rare anaemias, like for sickle cell disease or Gardos channelopathy, CoMMiTMenT can provide explanations how molecular defects result in altered cellular functions and finally in the symptoms of the disease. Based on these results, pharmacological targets have been identified and initially tested. First drug-concepts became available and were explored for personalised medical interventions by the clinical partners within the project or through a strong relationship with the European Network for Rare and Congenital Anaemias (ENERCA).

2 Summary description of project context and the main objectives

Anaemia, which is defined as a haemoglobin concentration less than 11–13 g/dl depending on gender and age, affects 1.6 billion individuals worldwide. Approximately 10% of these individuals are affected by rare anaemia (RA). This disease group includes approximately 90 different types of red blood cell (RBC) diseases, of which 80% are hereditary or congenital in nature. As the pathophysiology of the majority of these RA is poorly understood, the appropriate treatment is often ineffective or even lacking. Although the total number of affected individuals is substantial, the diversity in the underlying causes has resulted in limited interest from the pharmaceutical industry.

It is unacceptable to the CoMMiTMenT partners that the pain and suffering caused by clinically severe RA has been neglected as a result of lack of reliable high-throughput tools for scientific understanding and clinical diagnosis. The partners are committed to systematically working through the list of anaemia-linked medical conditions to generate knowledge that will lead to effective targeted therapies.

Recent studies indicate that several RA are associated with altered cellular ion homeostasis. These deficiencies may directly cause the disease, as in hereditary xerocytosis, overhydrated hereditary stomatocytosis, familial pseudohyperkalaemia, cryohydrocytosis and certain types of spherocytosis.

There are scenarios in which RA may be due to structural RBC abnormalities due to congenital membrane protein defects, e.g., hereditary spherocytosis, haemoglobinopathies (e.g., sickle cell anaemia, thalassemia) and glucose transporter GLUT1 mutations, which cause paroxysmal exertion-induced dyskinesias, inducing haemolytic anaemia by a cation leak.

A third category of RA associated with altered cellular ion homeostasis is enzyme deficiencies (e.g., phosphofructokinase deficiency). Moreover, it appears reasonable to assume that novel, unidentified ion homeostasis disturbances may significantly contribute to anaemia pathophysiology and may constitute an important group of undiagnosed cases of RA.

There is an increasing amount of evidence that disturbances of RBC ion homeostasis are the result of altered ion channel function. This group of so-called channelopathies is a heterogeneous group of diseases of which the molecular basis is, at large, unknown. Therefore, there are few or no treatments for patients who are affected by these disorders. Interestingly, several hereditary haemolytic anaemia are also associated with neurological diseases, many of which are known to be caused by channelopathies. However, this apparent relationship between the disorders remains unexplored. Therefore, studying disturbances in RBC ion homeostasis has the exciting potential of delivering targets for future therapies for not only rare types of hereditary haemolytic anaemia but also other non-haematological disorders. Moreover, the study of the pathogenesis of channelopathies will also be very important for exploring their potential involvement in the “undiagnosed RA”, which currently account for more than 20% of all RAs. To obtain access to a significant number of appropriate patients, CoMMiTMenT has ensured a strong collaboration with the European Network for Rare and Congenital Anaemias (ENERCA). This EU-funded international network connects experts and expert centres dealing with RA throughout Europe (www.enerca.org).

CoMMiTMenT will allow for the functional identification of channelopathies on a molecular level, i.e. the identification of malfunctioning ion channels or transporters that cause the disease or the associated symptoms. This approach is based on two complementary non-invasive microscopic methods. First, the dysfunctional channels will be identified and analysed as potential drug targets. There is a three-fold strategy in identifying such targets:

(i) some putative drug targets are based on previous work of CoMMiTMenT members. This is, e.g., the NMDA receptor (UZH) or the Gardos channel (USAAR).

(ii) Other drug targets can be extracted from the literature of the scientific community, e.g., the protein Piezo1 was identified to be mutated in patients suffering from hereditary xerocytosis. Since Piezo1 is a mechano-sensitive ion-channel it is a clear drug-target.

(iii) Further drug targets will be identified by molecular biology approaches of CoMMiTMenT members, for instance by next generation sequencing (UMCU).

In this manner, CoMMiTMenT contributes to the goal of the International Rare Diseases Research Consortium (IRDiRC) to deliver novel diagnostic modalities and 200 new therapies for rare diseases by 2020.

Despite common morphological features, circulating RBCs are a heterogeneous population of cells with markedly different properties, including their abundance and activity of ion transporters. This heterogeneity and the lack of tools with which to characterise specific sub-populations of cells for investigation represents a major challenge, as only a limited number of affected cells will be present in the blood sample of a patient with RA (generally in the range of 5–15%). It is therefore essential to identify the affected cells.

CoMMiTMenT developed a novel tool for the detection and characterisation of the vulnerable sub-population of RBCs in the blood of patients with RA. This objective has been achieved using the novel technique of MeCheM, which will be adapted for use with fluorescent molecular biomarkers (e.g., antibodies or fluorophores) and currently measures secondary cellular characteristics, such as the cell shape or other alterations of cellular morphology. Further, investigations can utilise a combination of microfluidics and imaging to positively identified cells that can be placed on an adhesive substrate, after which the surface topology of the cell can be determined using Scanning Ion Conductance Microscopy (SICM). The entire operation procedure of the µCOSMOS device chain consists of four distinct elements:

- MeCheM, in which cellular properties are imaged in real time, to allow for optical identification combined with microfluidics in red blood cell investigations. A range of microfluidic operations are available including an adherence test device that can capture cells through using tailored surface chemistries.• Scanning Ion Conductance Microscopy (SICM) results in an image of the surface topology and the identification of areas of increased electrogenic ion transport. This identification will be assisted by high-resolution optical imaging.
- Cell populations or selected cell can pass high or medium throughput automated patch-clamp recordings (SyncroPatch or Patch-Liner)
- The above measurements will be complimented with other established (but state of the art) techniques of the CoMMiTMenT members, such as next generation sequencing or FACS.

This procedure, which is based on the µCOSMOS device, will provide a novel and unique opportunity to locate and subsequently measure activity of ion channels, returning data regarding molecular structure and function. The rationale behind pharmacological interventions to correct channel abnormalities depends on the nature of the channel abnormality. If, as we expect in many of the rare anaemias, the abundance of the channel is the problem, like we could show for the NMDA receptor the pharmacological concept is the inhibition of the channel (memantine). If the structure of the channel (e.g. caused by mutations) is the cause for the abnormality, as we expect it for Piezo1 in xerocytosis an alternative to a channel antagonist is a pharmacological correction of the structure. This might even be the better concept (e.g. as it was shown

for the drug dantrolene which performed a „domain unzipping“ of the ryanodine receptor in certain patients suffering from catecholaminergic polymorphic ventricular tachycardia (CPVT)). Yet another scenario is that channel activity is increased due to the increased abundance of an agonist in the blood or gain of function mutations (e.g. the Gardos channel in the Gardos Channelopathy), which requires a pharmacological concept to either limit the „production“ of the agonist or to trap it or a drug to maintain/limit channel activity, like senicapoc for the Gardos channel. To summarize, there is not a single pharmacological intervention concept to follow. CoMMiTMenT improved the understanding of the molecular and functional background of the particular channelopathies to develop tailored pharmacological interventions. We are at a more advanced level for the NMDA receptor in sickle cells but also for other rare anaemias we identified concepts, like senicapoc for treatment of the Gardos channelopathy.

The functional molecular readout has multiple applications:

i. The identification and characterisation of the affected channel(s) that can be used as biomarkers in patients with RA;

ii. The development of pharmacological interventions to correct channel abnormalities. These interventions serve as the basis for applying and exploring potential treatments (personalised medication).

The integration of the above-described imaging technologies (MeCheM and SICM) into a device chain (µCOSMOS) was applied to study RA with known and unknown pathophysiologies and/or phenotypic expressions. The patients were identified by the project's clinical and biomedical partners using conventional technologies (e.g., molecular biology, flux measurements, and osmotic gradient ektacytometry) or by other available and novel approaches (e.g., nanoparticle tracking analysis).

The identification and characterisation of specific alterations in abundance and function of ion transporters involved in the channelopathies such as hereditary xerocytosis, overhydrated hereditary stomatocytosis, familial pseudohyperkalaemia, cryohydrocytosis, certain types of spherocytosis, hyperphosphatidylcholine haemolytic anaemia, sickle cell anaemia, thalassemia syndromes, and RBC enzymopathies (i.e., disorders of glycolysis and glutathione metabolism) is intended to lead to the development of novel diagnostic and prognostic tools and new medications. All of the above-mentioned diseases and pathologies have been investigated by closely collaborating clinicians and basic scientists within CoMMiTMenT. The path forward is: (i) the identification of the subpopulation of cells with altered ion homeostasis using fluorescent dyes; (ii) the functional characterisation of altered cellular conductance; (iii) parallel molecular biology approaches to identify the dysfunctional channel; (iv) the development of an "easy" diagnosis procedure.

This project resulted in substantial knowledge gain and success with respect to both the identification of diagnostic targets and the development of drugs and personalised medication (clinical trials are ongoing). Furthermore, the technological concepts were developed and used, CoMMiTMenT contributed to the overall aim of IRDiRC to deliver novel diagnostic modalities and 200 new therapies for rare diseases within the duration of the project. For example, partners of the consortium identified the NMDA receptor as a source of Ca^{2+} entry into RBCs in sickle cell disease. The obtained data suggested the active participation of this receptor in decreased deformability and enhance adherence of RBCs of sickle cell disease patients favouring haemolytic and vaso-occlusive crises. The already approved antagonist that blocks NMDA receptor-associated channel activity (memantine – approved to treat Alzheimer's disease) rapid clinical

trials of memantine in the implementation of this treatment for sickle cell disease. CoMMiTMenT accompanied a phase II clinical study (performed and funded outside of CoMMiTMenT), in which the concept of personalised medications was tested. This test will was based on the µCOSMOS technology developed within CoMMiTMenT and publication is in preparation.

In summary, CoMMiTMenT developed and validated new technologies and new methods to be combined in a novel diagnostic device chain. Individual imaging technologies, and to a larger extent the unique fusion of technologies, allowed for the identification and probing of the molecular players that underlie rare anaemia. In doing so, this technology will provides a novel diagnostic tool and a better understanding of the underlying pathophysiology of rare anaemia for several anaemias like sickle cell disease and Gardos channelopathy.

3 Description of main Scientific & Technological results/foreground

3.1. Work Package 2 – Specifications of imaging technologies

Main objectives of WP 2

O2.1: Define initial specifications for application, hardware, software and the µCOSMOS device.

O2.2: Refine and evaluate specifications by several feedback loops during the project.

Activities and results of WP 2

During the project period the project partners have worked on the specifications of the µCOSMOS system, the proposed instruments and the required software, hardware and analysis tools. Several meetings (e. g. consortium progress meeting in London, individual partner meetings) were held and major results could be achieved within WP2:

1. The requirements for the planned µCOSMOS system were discussed and refined with all the partners. We decided to use a loosely-coupled architecture for integrating the µCOSMOS hardware devices.
2. New microfluidic chip designs were proposed by Epigem (P8) and UZH (P2) that provide additional analytical methods and capabilities for the µCOSMOS system (see T2.1).
3. For integrating the result data of each individual device, a central data management system was proposed and described within the specifications of the µCOSMOS system.

Furthermore, the requirements for image analysis methods were refined in many bilateral sessions between the project partners. This is reflected by the design of the central data management system which provides specific support for image data and flexible offline image analysis tasks.

The final specification of each µCOSMOS device and the integrating architecture is described in D2.3. The original objective was for the OMiCS and SICM operation to be done in series, possibly automatically, and the title (µCOSMOS), where the "µ" indicates the scale of cellular dimensions, was proposed for the combined microscopes. Instead a better, more flexible final specification for µCOSMOS has been arrived at (see Figure 1).

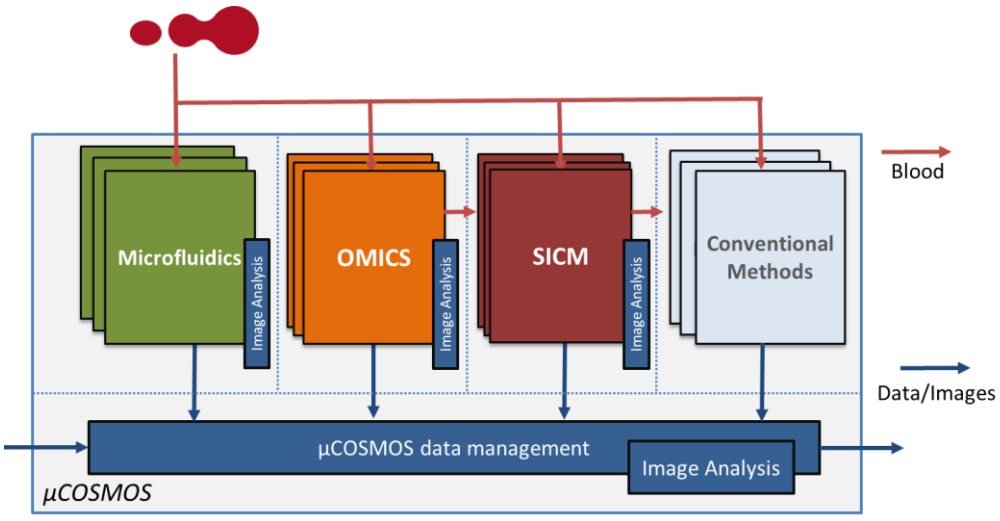


Figure 1: µCOSMOS architecture

In addition to OMiCS and SICM, a set of advanced multifunctional microfluidics tools (MeCheM) have been developed that meet additional clinical requirements and proof of principle work using patient samples has shown clinical usefulness. It has been shown that the microfluidics apparatus can, for example, perform mechanochemistry, as part of a range of microfluidic RBC sample test kits containing reagents and plug and play consumable components; that can be used with reusable sample input components (pumps & valves) as well as utilizing microscopy hardware and image analysis software. Described here for the first time is proof of principle of a set of chips for mechanical deformation (MeCh), chemical alteration using added chemicals (CheM) and the combination of the two (MeCheM) where the mechanochemical change results in, for example, RBC adherence changes (cell-cell and/or cell-surface). The observed morphology of the cell population are characteristic of the RBC type (diagnostic) and further information can be obtained from the kinetics of the induced processes. An initial set of SOPs related to the use of these devices has been developed in combination with planned instrument design improvements and end user preferences.

We have concluded that a single instrument does not provide the flexibility required to perform the range of measurements needed for full characterisation of RBCs in the blood of patients. The package of measurement solutions now envisaged, partly based on a results from blood samples taken from a cohort of patients ("customer driven" one could say) have been arrived at by integrating the expertise of the SME partners in a number of ways that are described in this Deliverable. For example, Arivis image analysis software is being used in both OMICS and MeCheM, with Epigem also making the microfluidic devices for both; and MeCheM adherence devices can be used as the input for SICM.

Setting the right specifications for the µCOSMOS was an essential prerequisite for the success of the project. Within this Work Package all partners have worked on multiple revisions of a specification document (Deliverable 2.1, 2.2, 2.3) and have furthermore worked on refining these specifications within WP3-6. The specification starts with the analysis of general and application specific requirements (User groups and workflows, relevant rare anaemias), that are used to specify the design of the prospected technical devices and the integration layer.

User groups and workflows

First, we analyzed potential user groups and distinguished between clinical and research users. These user groups have different expectations and requirements that need to be met by the system's specification. Additionally, different workflows were identified and discussed by the partners. The workflows mostly differ in their focus on (a) explorative research or (b) strict diagnosis tasks.

Relevant Rare Anaemias

The main task of the project is the support of diagnosis of patients affected with a rare anemia in which routine lab methods have not been useful to differentiate among different disease. Currently, around 15% of rare anemia patients are undiagnosed or misdiagnosed. Reticulocytes, blood transfusion and a small percentage of cells with morphological changes hamper an appropriate diagnosis in many cases. The most probable cause of these undiagnosed rare anaemias is an ion disturbance due to (a) a channelopathy or (b) to a structural membrane alteration.

The main task of the µCOSMOS system is to support a novel workflow for the characterization of rare anaemias. The following list of specific parameters is required by the medical partners and can be generated by the prospected technical methods of the µCOSMOS system:

- RBC shape and morphology
    - Classification of morphological types (see Figure 2)
    - Symmetry of cells

- Surface topography
- Quantification of blood composition

- Structural surface calculations of individual cells in µM2
  - RBC vesicle analysis (biomarkers, high resolution, surface)
  - Aging and clearance markers (band 3 clusters, annexin binding, membrane-bound damaged Hemoglobin (Hb) products)
  - Adherence
  - Fluorescent markers (Ca2+-content, pH-Value (both before and after stimulation), antibodies, imaging techniques)
  - Membrane conductance
  - Cellular (deformability) behavior in small capillaries and under hypo- and or hyperosmolaric conditions
  - Evaluation of reaction of RBC to different stimulation conditions
  - Sorting and separation of single cells and cell populations for in-depth analysis (by other parameters)

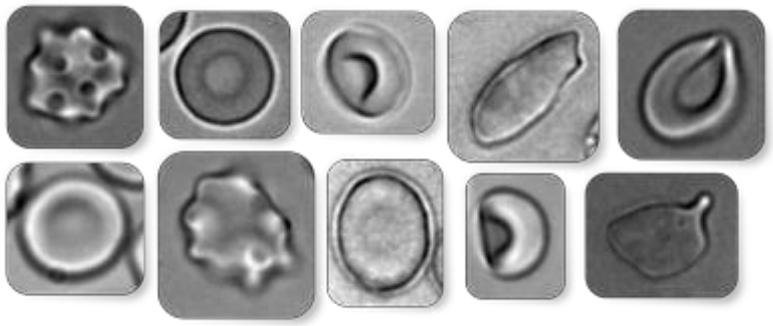


Figure 2: Variations in Morphology of RBCs

Furthermore, many additional parameters - acquired by established, conventional lab based methods - need to be integrated into the µCOSMOS software environment. They should be associated with information about patients and the corresponding blood samples. This enables a combined analysis of all corresponding experiment or analysis results. The data models for patients, blood samples and the models for more than 30 conventional analysis methods have been defined by the partners (see Deliverable 2.2). This influences the architecture and design of the µCOSMOS software.

Several technical methods will be combined in the µCOSMOS system. Each of the core technologies was further specified and lead to the specification of the software integration layer.

OpenIO Labs: SICM based methods

SICM is a state-of-the-art nanometre imaging system. It comprises a scan head, a controller, and OpenIO data-acquisition systems. The robust mechanical design of the Ionscope SICM ensures high precision measurements. It can be used as a standalone system or integrated with an inverted light (or confocal) microscope. The Ionscope software offers a variety of supported modes and in-built system functions such as automated immersion, surface detection, real-time ion current display, real-time 2D and 3D display of images as they are formed (see Figure 3). This unique feedback mechanism allows the nanopipette to avoid physical contact with the sample – meaning that SICM is particularly well suited for imaging soft samples such as live cells. SICM nondestructively images convoluted features that other systems would damage.

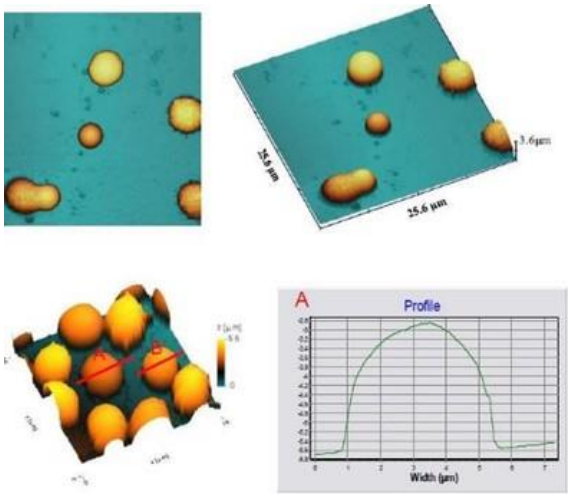


Figure 3: SICM surface scans

OptoRobotix: optical cell sorting methods

The "Optofluidic Microscopy-based Cell Sorting" (OMiCS) system is an optics based cell sorter that uses image processing to identify cells of interest and laser beam shaping to isolate these identified cells as they flow in a microfluidic environment. It works similarly to a quality control stop in a production line wherein products circulated by a conveyor belt are inspected by a specialist, or a computer and the defective ones are removed.

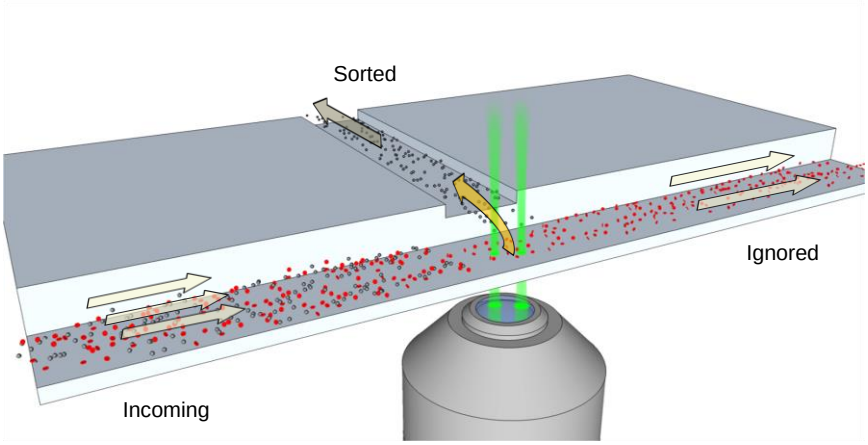


Figure 4: OMiCS principle

Epigem: multifunctional microfluidic methods

The sample preparation requirements of the CoMMiTMenT end users are diverse but can be accommodated using a portfolio of microfluidic devices that can be used within "A Common Product Architecture". This has been the focus of attention for Epigem since D2.2 and we believe a viable solution has been found that brings together the sample preparation requirements of OMiCS and SICM suitable for validation work in WP6.

Secondly it has become clear that to understand red blood cells and to use that understanding as a diagnostic tool the key chemistry is "mechanochemistry". Epigem have been taught (mainly by UZH) that mechanochemistry can be used diagnostically in the case of rare anaemia identification and treatment monitoring and that it is likely that mechanochemistry is the chemical transformation process from reticulocytes to erythrocytes during red blood cell synthesis.

Accordingly, Epigem has designed and fabricated a range of microfluidic devices that enable interrogation of red blood cells using mechanical forces alone, chemical forces alone or both simultaneously at the same time as enabling chemical surface interactions including molecular and cell recognition not unlike immunoassays. For reference simplicity and possibly branding purposes we are calling these "MeCHeM: RBC analytical test methods using mechanical deformation and chemical modification".

Software Architecture

A central µCOSMOS Data Management component is a web accessible application that provides functionality to manage and integrate the analysis results of µCOSMOS devices. All the technical devices combined in a µCOSMOS installation require different configuration and create a multitude of different quantification numbers, analysis results or reports and many external data fields need to be associated (see requirements).

The central management component should use a very extendible data model (e. g. based on JSON and JSON Schema): For each single data model (see D2.2 appendix) a JSON Schema was generated. The µCOSMOS management software can register single schemas, associate them with specific experiments/blood samples/patients and automatically create an easy-to-use web interface for editing specific schema instances. This will be embedded into an easy-to-use and workflow oriented solution.

Furthermore, advanced reporting and export methods are required that include relevant data and allow new investigations by combining and analyzing results of additional statistical or technical methods.

To support the data flow in the µCOSMOS device the following requirements for the central management component can be specified:

- General control and integration software for technical methods and devices
    - Provide user open interfaces for controlling and configuring hardware modules
    - Standardized data formats for result and configuration exchange
    - User Guidance by Software supported SOPs (Workflows)
- Management of Experiments
    - Management of patients and all relevant data.
        - Anonymized patients and sample data
        - Support for control groups/samples
        - Compiling data from devices along the µCOSMOS workflows
        - Flexible Integration of external analysis results (Include conventional measurements of already established lab methods)
    - Management of µCOSMOS workflows
        - Reflection of µCOSMOS structure
        - Configuration of required technical modules
        - Easy-to-use definition of Workflows, SOPs
        - Support for repetitive analysis and stimulation experiments
    - Reporting and Export of experiment and workflow data
    - Archiving
    - Advanced statistical, analytical methods on data (data warehouse)

Software integration of image analysis functions

Within the project the technical methods (OMiCS, SICM, MeCheM) are combined with advanced image analysis functions that create numbers and features based on acquired image data. In contrast to the already mentioned management methods the demands for this software interface and integration are

different. Especially for the flow based optical sorting methods in the OMiCS device, a high performance image analysis is required that analyzes large amounts of data very fast to provide results within a certain time frame.

In discussion with the partners arivis proposed to use high performance C++-interfaces and provides a library to embed and execute arivis AnalysisPipelines within a custom application. The advantage of this approach is the possibility to comfortably and interactively define these pipelines for different purposes in the desktop tool arivis Vision4D. After exporting these pipelines they can be used to configure the C++ analysis library and execute this analysis on different datasets.

3.2. Work Package 3 – Proof-of-principle: Application of optofluidic microscopy for RAs

Main objectives of WP 3

O3.1: Design and production of microfluidics, image processing algorithms and optical hardware.

O3.2: Integration of microfluidics, image processing and optical hardware.

O3.3: Characterization, specification and validation of OMiCS platform.

Activities and results of WP 3

Objective 3.1

Optorobotix Aps has designed a microfluidic system suitable for OMiCS and has made a first fully functional prototype of the optical hardware. For the detection of cells, basic image processing algorithms have been integrated in the system and this work is ongoing to include more recent techniques from computer vision or machine learning. Optorobotix Aps has successfully integrated microfluidics, basic image processing, and optical hardware. Several optical beam shaping techniques have also been developed to improve the catapulting response of the detected cells within the microfluidic flow chambers. The techniques developed include Holo-GPC and geometrically shaped point spread functions (PSFs) that both take advantage of phase only spatial light modulators (SLM's) that are efficient in reshaping laser light. The programmatically shaped light is then used to exert optical forces on microscopic particles such as red blood cells based on the optical tweezing and manipulation principles originally invented by Arthur Ashkin at Bell Labs., USA that subsequently received the Nobel Prize in Physics (2018).

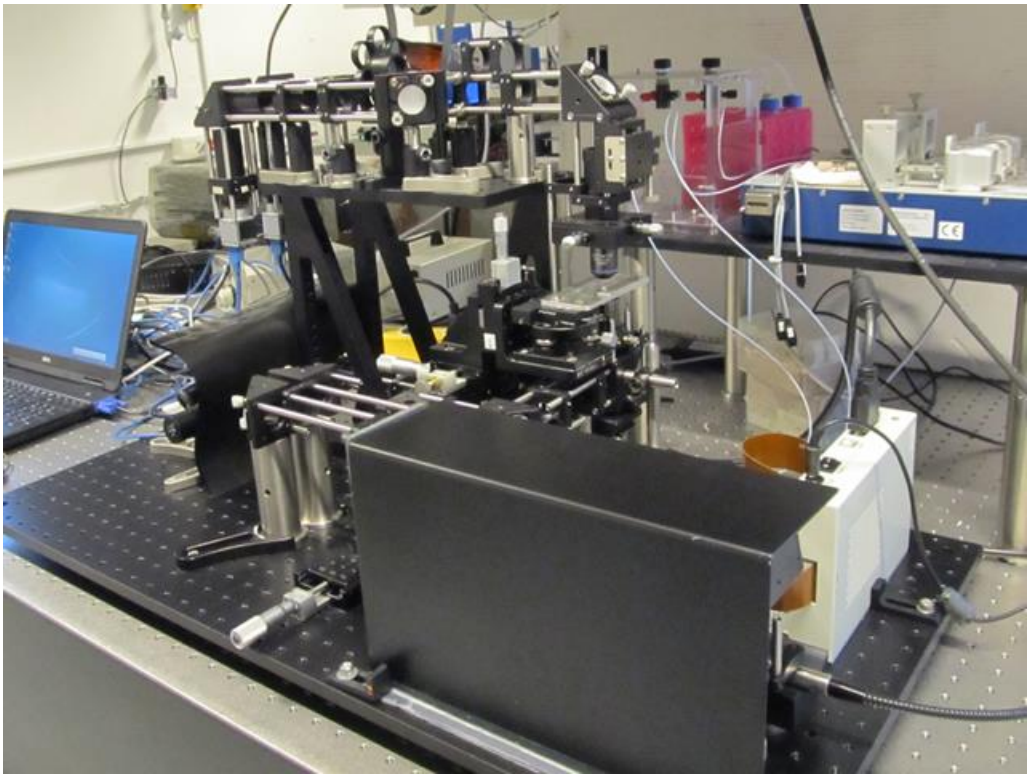


Figure 5: The OMiCS hardware.

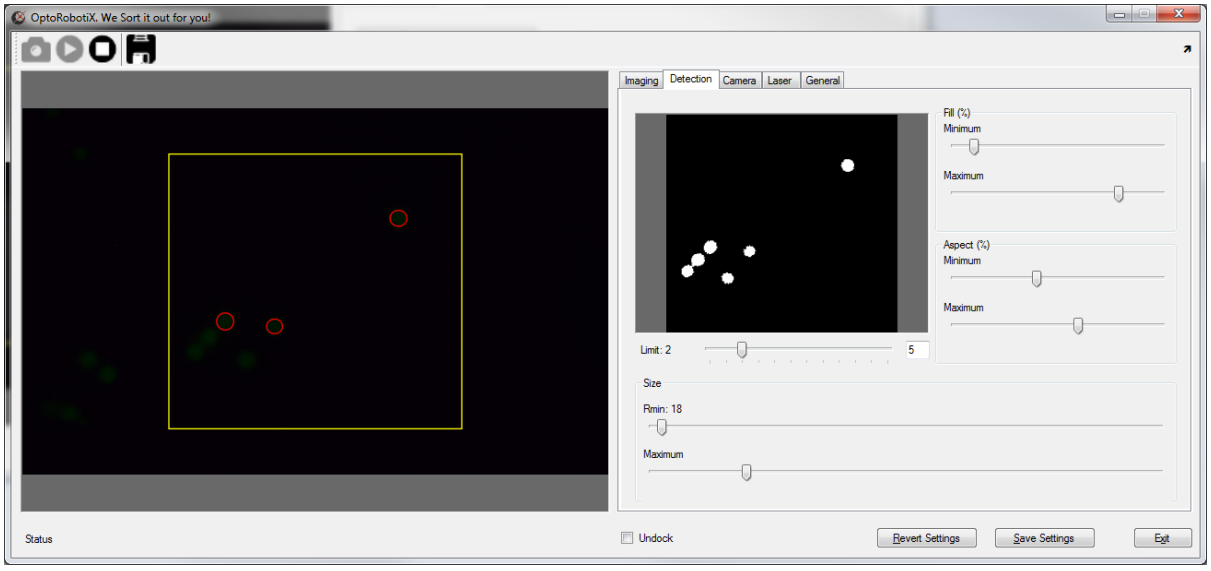


Figure 6: Fluorescence imaging and software object detection in the OMiCS.

Objective 3.2

The integration of the software and imaging hardware has been done in cooperation with arivis AG. Optorobotix Aps has also used its in-house software utilizing a freely available image-processing library to deal with other hardware constraints specific to the OMiCS. Under gentle flow configurations, the OMiCS has demonstrated sorting of RBCs on chips designed and fabricated by Epigem Ltd. Several iterations of the microfluidic chip have been tested for advanced flow control. But ORX eventually returned to the original two-channel cross flow concept, but with an important change of integrating the reservoirs into the chip using the MeCheM priming system to prevent bubble trapping problems as suggested by University of Zurich. In addition to the cross-flow approach, a single channel chip wherein cells are catapulted into an adhesive ceiling has also been investigated during the trial at Saarland University.

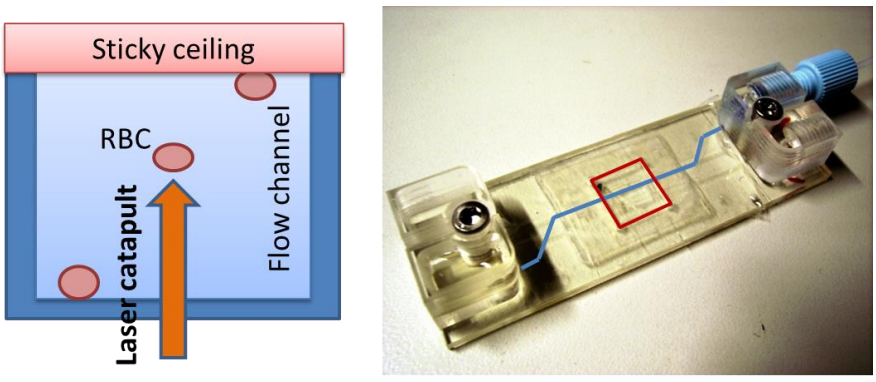


Figure 7: A microfluidic chip wherein cells are to be catapulted into an adhesive ceiling for subsequent collection.

Improvements in the integrated imaging have also been done. An alternate fluorescence imaging channel has been incorporated in the OMiCS. This allows users to take advantage of the more familiar fluorescence techniques and can supplement the performance of image detection.

Objective 3.3

Optorobotix Aps has run characterization and basic validation of the OMiCS with whole blood from healthy persons as part of fulfilment of objective 3.3. This has been met with several challenges due to the control of the flow and the low refractive index contrast of the cells and the surrounding fluid medium.

Technical challenges were met when characterizing the OMiCS platform and this has motivated Optorobotix Aps to propose new light shaping modalities and disruptive Light Robotics solutions.

Several laser beam shaping techniques that exhibit an extended propagation distance while still being relatively easy to operate have been developed: The first being Holo-GPC which combines advantages of holography, originally used with the OMiCS, and GPC. Despite Holo-GPC's advantageous features, it has shortcomings such as additional fabricated precision components and difficult alignment, especially for non-optics users. An extension of holography that circumvents the additional requirements and constraints of Holo-GPC has been subsequently developed. The new methodology platform presented based on "geometric mapping" has the advantage of not requiring the additional components and inherent alignment issues of Holo-GPC, while also exhibiting the desired extended propagation profile of the generated laser light distributions.

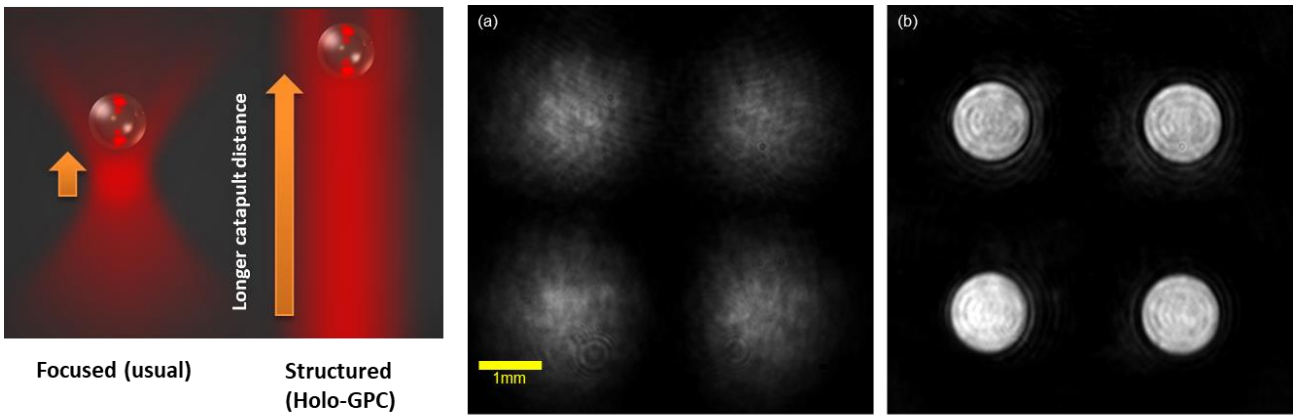


Figure 8: Structured light fields that can be produced by Holo-GPC or Geometric beam shaping that allow extended axial catapulting of cells.

Dissemination

The published papers have already generated substantial activity in the related scientific community triggering other research groups to follow-up on the results. To overcome the technical challenges met in the activities and for documentation and dissemination, Optorobotix Aps has been able to publish the following during the last two years of the EU-project. Moreover, a comprehensive Elsevier book volume (ca. 485 pages) on "Light Robotics" was published in summer 2017 and is today the 2nd most popular Elsevier book in their nanophotonics series according to the series editor:

- "Holo-GPC: holographic generalized phase contrast." *Optics Communications* (2017).
- "Light Shaping with Holography, GPC and Holo-GPC." *Optical Data Processing and Storage* (2017)

- "Light Robotics: a new technology and its applications." *Fourth Annual Conference on Optical Nanospectroscopy*. 2017.
- "Cell growth regulation studies on our biophotonics workstation." *Complex Light and Optical Forces XII*. Vol. 10549. International Society for Optics and Photonics, 2018.
- Software for Real-Time Light Shaping and BioPhotonics Applications." *Complex Light and Optical Forces XII*. Vol. 10549. International Society for Optics and Photonics, 2018.
- "Light robotics: a new field of research." *Complex Light and Optical Forces XII*. Vol. 10549. International Society for Optics and Photonics, 2018.
- "Three-dimensional light sculpting using a geometric analysis," Optics Communications 431, 21-215 (2018).
- "Point spread function shaping using geometric analysis," Optics Communications 427, 522-527 (2018).
- "Optical cell sorting with multiple imaging modalities", Complex Light and Optical Forces XI 10120, 1012018 (2017)
- "Cell sorting using efficient light shaping approaches", Complex Light and Optical Forces X, 9764, 97640F (2016)

3.3. Work Package 4 – Proof-of-principle: Application of ion conductance microscopy for RAs

Main objectives of WP 4

O4.1: Design of a SICM microscope ready for integration with OMICS platform and with microfluidic stage for sample measurement; development of image processing algorithm.

O4.2: Integration of SICM microscope with OMICS platform and microfluidic stage for sample measurement; integration of image analysis algorithm.

O4.3: Characterization of the system using RBCs from RA patients.

Activities and results of WP 4

Hopping mode scan of SICM

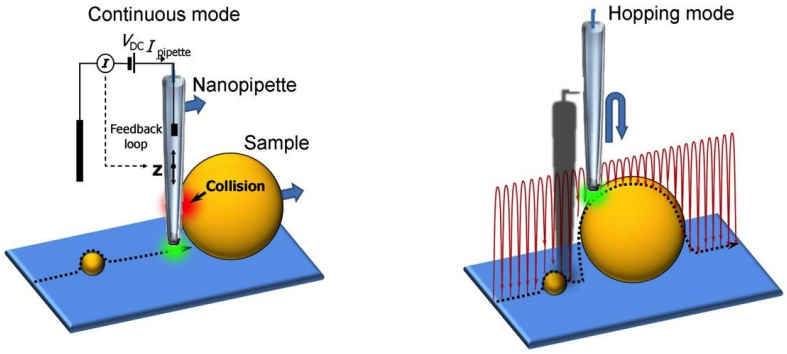


Figure 9: Illustration of traditional continuous mode and hopping mode (Novak et al., Nature Methods, 2009)

Scanning Ion Conductance Microscope (SICM) uses a glass nanopipette to follow the sample surface in a non-contact way. The distance-dependant ion current is used as a feedback signal to controller pipette position in Z direction while it raster scans in X and Y directions. A 3D topographical image can be generated to reveal the surface structure and can be used for further operations of the nanopipette. SICM is the best technique for scanning Red Blood Cells, due to its non-invasive manner that can repeatedly scan the same soft and living RBCs.

In Ionscope SICM, a hopping mode scan is used that allows nanopipette to scan a tall sample without any collision with the cells. In hopping mode, pipette approaches to the sample surface until a predefined current decrease is reached, then pipette withdraw before moving in XY directions. However, in scanning RBCs, the cells height still causes a challenge in SICM. Normal RBCs have a height of 6 to 8 μm, there is an abrupt change in height when pipette scans from the bottom dish to the cell, which requires a very big hop height to avoid collision, but at the same time, big hop height significantly increases the scanning time. There are several methods implemented to increase the speed, which is discussed in the next session.

Optimisation of scan conditions and parameters

Cell detach issue and scan speed

Cell detach issues were found in some experiments with cells in Petri-dish, that it is difficult to complete a scan without cells moving. Therefore, it was decided that using a microfluidic system for SICM was not

likely at this stage. SICM was then used as a standalone system to analyse the RBCs. Cell-Tak, a cell adhesive, was used to attach the cells on to the Petri-dish. However, in more regular use, RBCs were still found moved away from surface during a scan. Also, as in Figure 10, cell morphology changed with Cell-Tak coating.

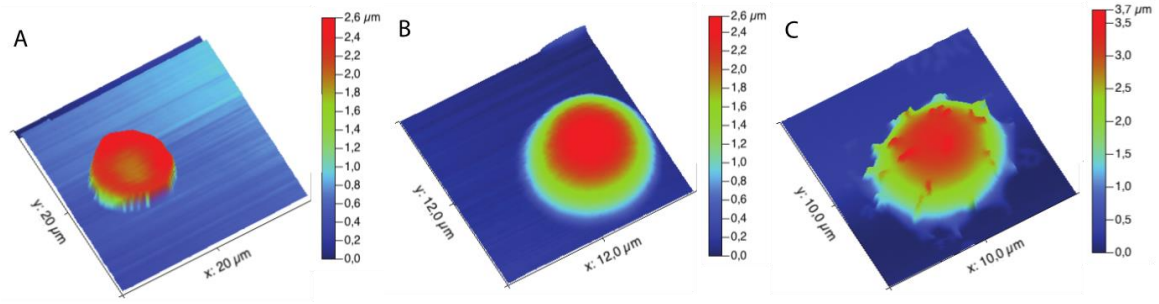


Figure 10: Topographic scans of human red blood cells in physiological solution. A: Discocyte, B: Spherocyte, C: Echinocyte. Scans B and C were performed with coated Petri dishes, Scan A was performed without coating.

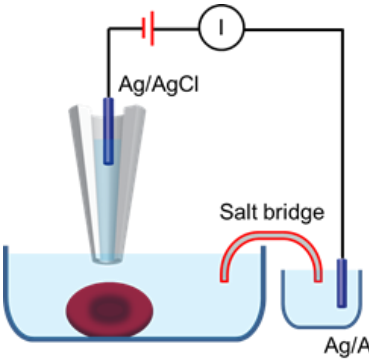


Figure 11: Modified SICM configuration for RBC scanning with a salt bridge and reversed bias.

According to the paper (Zhu, C., Shi, W., Daleke, D., & Baker, L. A. 2018), there is possible Ag^+ leak from electrodes to interfere with live RBCs. In the new SICM setup, an agar salt bridge is added to separate the reference electrode from the red blood samples and the pipette bias is also reversed from + to – to stop the Ag^+ leak. Ag^+ might be one of the reasons that cells detach from the Petri-dish during a scan.

Scan parameters are the main reason that cells detach during a scan. When the pipette scans from the Petri-dish surface to the RBC, there will be an abrupt change in height, if the parameters are not appropriate that pipette could move fast enough to follow the cell surface, it will crash into the cell and drag the cells off the surface. To make sure that the pipette can respond to the sudden change in height, several changes in parameter settings were suggested: increase the minimum hop height, decrease the fall rate and more image points. However, this ends in long scanning time, as in Figure 10, such images take 1 hour to obtain. Also the change in height from Petri-dish to RBS in is the order of 5 micrometres, but the features on RBC surfaces can be in the order of 50 nm to a few hundred meters. The same parameters cannot both provide a safe scan for RBC edges and image the small features on the RBC surface. Therefore, a protocol including a first scan to obtain a general shape and location and a second scan only on RBC top surface was used.

For living cells, they were incubated in the Petri-dish for at least 30 mins to 1 hour without any adhesive. Petri-dish was then washed with PBS 3 times, the solution was added gently from the wall not directly to the attached cells. For fixed cells, the Petri-dish is coated with Corning Cell-Tak adhesive and the cells were

then incubated in room temperature for 30 mins. It is beneficial to have high cell density. Observe the cell density with optical microscope and add more if the density is low. If the cell density if too high, the unattached cells will be washed away.

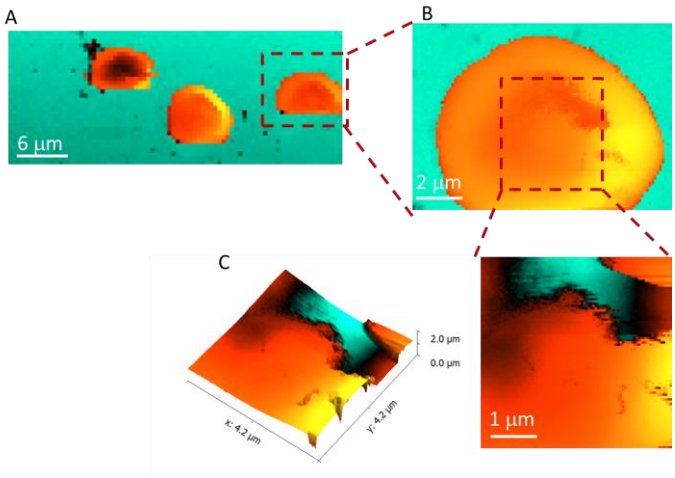


Figure 12: A. a fast low resolution scan was performed to identify a cell of interest. B. a higher resolution scan of a single RBC. C. a scan on the surface only.

Here is an example scan of a fixed cell (Figure 12). Step1: a large scan is used to help select a cell of interest. Step 2: a scan of the single cell was performed to identify interesting structures. Step 3: a higher resolution of just the surface structure was scanned. It has to be mentioned that it is possible to move the pipette directly onto a RBC to study the surface structure, if the feature is common on each cell.

Optimum scan parameters have been found for RBC scans. Minimum hop height (MHH) is the most critical parameters. During the scan, the first line has a very large hop height, because the sample height is unknown. In the second line, SICM uses the information from the first line to predict the height of the sample, pipette moves to that height plus a minimum hop height. For tall cells (~8 µm), 1 µm is needed. Smaller hop height with 800 nm, 500 nm were also used for shorter cells. It is advised to start with 1 µm, and check the image to decrease or increase the hop height. If a small scan is performed on just on the cell surface, 200 nm or less can be used.

For a larger area 40 µmX40 µm, 1 µm/pixel or 500 nm/pixel were used to get a low resolution, which allows visualisation of all cells in that area and takes around 5 mins for each scan. Decrease to 200 nm/pixel or 100 nm/pixel when scan a smaller area or higher resolution is needed.

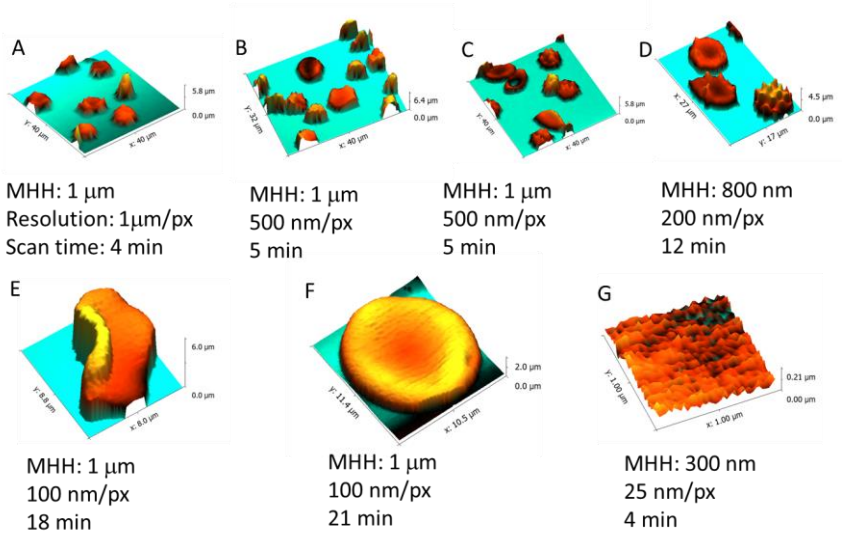


Figure 13: Scan time comparison with different minimum hop height and resolution settings. A~D: large area scans. E~F: single cell scans. G: surface scan.

From Figure 13 A&B, the scan time is not significantly increased when the resolution is doubled. This is because the first scan line used most of the time because the large and safe hop height. Figure 13 G is an example of a high resolution scan on a small area of the cell surface. It has to be mentioned here that the small features here are artefacts in the scan instead of real surface features.

Characterisation of RBCs

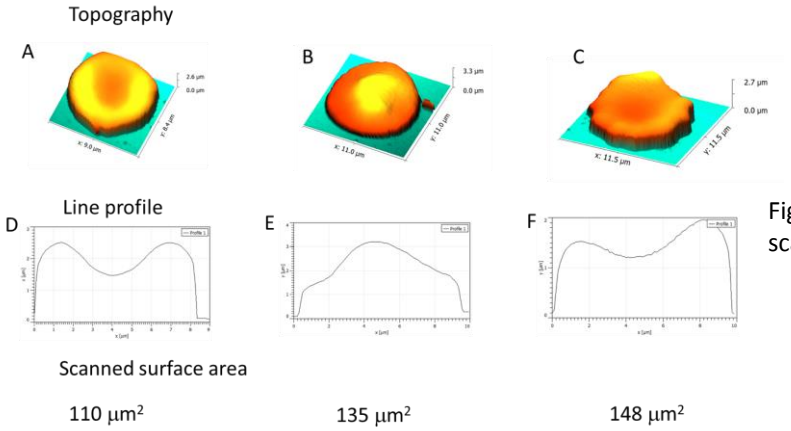


Figure 14: Topography, line profile and scanned surface area of RBCs.

SICM topography provides a 3D visualisation of the cells, therefore the line profile and scanned surface area can also be extracted from the images to understand the differences in cell shapes.

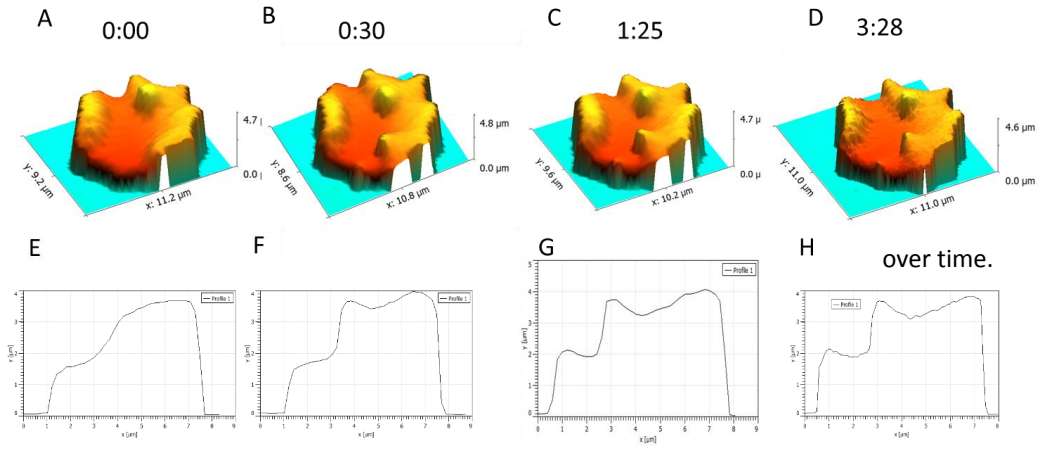


Figure 15: Repeated scans of the same cell

Morphological changes with time can also be recorded with SICM on living cells. The repeated scans with SICM also demonstrated that the new protocol fixes the cell detachment issue. This also indicates SICM can be a powerful tool to monitor cell dynamics.

Deoxygenated RBCs were analysed by SICM. They are one example of extremely distorted RBC shapes. They vary significantly in shape. With the current protocol, the parameters are suitable for both normal and extremely distorted cells.

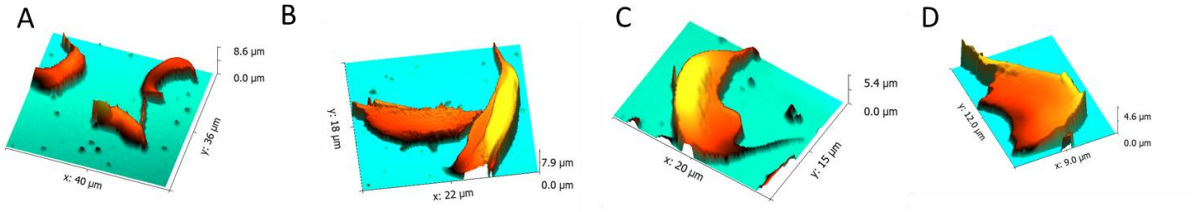


Figure 16: SICM topography of deoxygenated RBCs.

Integration with light microscope and patch clamp

In addition, the SICM setup was combined with a fluorescence microscope. The idea behind was to simultaneously scan the surface of the cell and have a look at the intracellular calcium content of the cell. This could be useful, when working with stimulating or inhibiting agents, which influence the calcium content of RBCs.

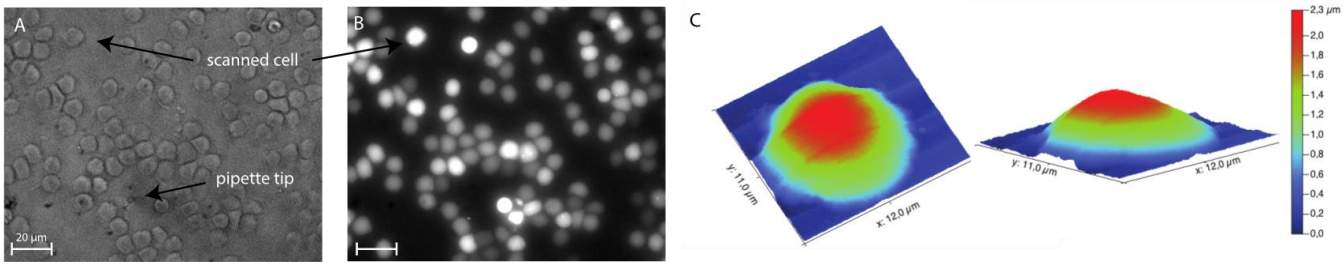


Figure 17: White-light (A), fluorescence (B) and topographic (C) images of human red blood cells. Cell was scanned in physiological solution after loading with Calcium dye Fluo4.

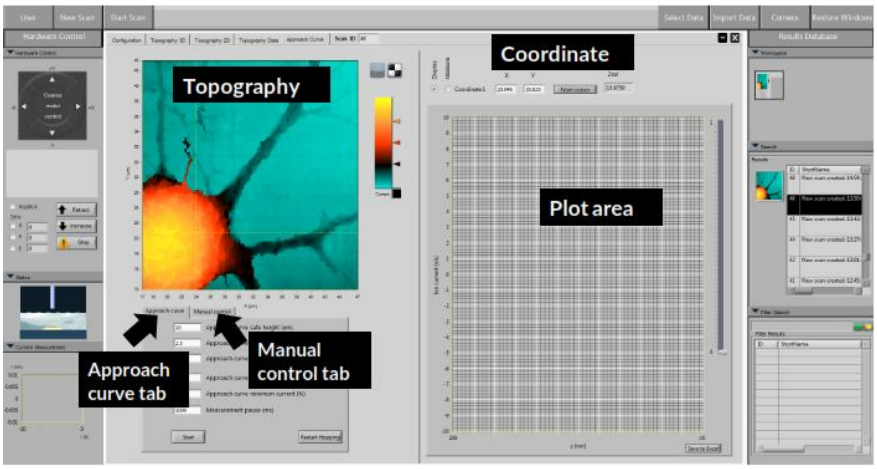


Figure 18: Screenshot of the software with Manual controls.

In the SICM software, there is also an approach mode and manual mode, which allows manual control of the pipette to any point on the scanned sample.

The user maps the cell surface in a standard SICM topographical scan. Once that 3D information is in the SICM software's data base it can be used to guide the pipette to any point on the measured surface. This can be used for a targeted patch clamp. Pipette can be used to disturb the cell and/or measure the ion channel activities. This can be useful for further studies on piezo channels discussed intensively in this project.

3.4. Work Package 5 – Treatment concepts for personalized medicine

Main objectives of WP 5

O5.1: Characterization and categorization of the types of RA that are associated with altered ion homeostasis.

O5.2: Delineation and detailed analysis of the different molecular players involved in altered cellular ion homeostasis.

O5.3: Defining the molecular targets for potential pharmacological interventions.

Activities and results of WP 5

1. Characterization of the selected RAs.

Characterisation of RAs was performed using samples obtained from the haematological clinics at the sites of UMCU and IDIBAPS as well from Dr Paola at Bianchi Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, UO Oncoematologia, UOS Fisiopatologia delle Anemie, Milan, Italy. We also gained access to the Clinic of Pediatric Hematology at the HaEmek Medical Center, Afula, Israel, where the MeCheM red blood cell analyser is on trial from February 2018 onward. We have obtained samples of patients with β-thalassemia major/intermedia/minor, sickle cell disease spherocytosis, xerocytosis, Gardos channelopathy, and enzymopathies. A total of 121 individuals (78 patients and relatives and 43 controls) from UMCU (91) and IDIBAPS (29) has been characterized with the already set up laboratory tests in UMCU, UZH, USAAR and IDIBAPS/IJC. Breakdown of disease category is shown in the table below:

Table 1: Breakdown of disease category

Controls	43
Patients	78
Hereditary spherocytosis	23
Hereditary elliptocytosis	1
Rare constitutional hemolytic anemia due to a red cell membrane anomaly	2
6-phosphogluconate dehydrogenase deficiency	4
Alpha-thalassemia	4
Dehydrated hereditary stomatocytosis	3
Gamma-glutamylcysteine synthetase deficiency	1
Hemolytic anemia due to glutathione reductase deficiency	1
Hemolytic anemia due to red cell pyruvate kinase deficiency	4
Hereditary methemoglobinemia	1
Rare anemia	34
Total	78

1.1 Osmotic Gradient Ektacytometry

IDIBAPS /IJC carried out a study to assess the usefulness of new generation osmotic gradient ektacytometry (OGE) for measuring red blood cell deformability and therefore for the diagnosis of red blood cell membrane disorders. A total of 75 cases of hereditary spherocytosis (HS), 9 hereditary elliptocytosis (HE), 5 hereditary Xerocytosis (HX), were analysed with osmotic gradient ektacytometry in addition to the routine red blood cell laboratory techniques. The most robust osmoscan parameters for HS diagnosis were determined using receiver operating characteristic curve analysis. Accordingly, when compared to the mean of normal controls, the best diagnostic criteria for HS were the combination of decreased minimal elongation index up to 3% and increased minimal osmolality point up to 5.2%. Using this established criterion, for the diagnosis of HS, OGE reported a sensitivity of 93.85% and a specificity of 98.38%.

1.2 Genetic characterisation

IDIBAPS has developed a genetic panel in collaboration with CNAG (National Genomic Analysis Center). This panel include 33 genes already identified as cause of haemolytic anaemia and haemoglobin as reported before, and also 35 genes involved in haemolysis modulation, homeostasis modulation, membrane stability and erythropoiesis modulation that could be causing rare anaemia with unknown origin or modifying the clinical manifestations of patients with known haemolytic anaemia. IJC has continued the inclusion of patients for genetic characterization and has led the analysis of variants for both genes leading to RAs and modulators of clinical manifestations. Genetic characterization using NGS panel by IDIBAPS /IJC have demonstrated that there is a high genetic heterogeneity in patients with RBC membrane disorders, as almost each family carries a unique mutation that is not observed in any other no related family. The present study reveals the usefulness of Next Generation Sequencing (NGS) panel, which allows the molecular diagnosis of almost the 90% of the patients and it would avoid misdiagnosis that could lead to splenectomy in contraindicated cases such as in hereditary stomatocytosis (HX). Moreover, the 11% of undiagnosed patients will be analysed through a second NGS gene panel including potential new genes leading to chronic haemolysis and/or sequenced by whole exome sequencing with the aim to identify new disease-causing genes.

NGS Gene panel analysis included in their genetic panel study 233 samples from 123 different families. 165 of these are patients presenting clinical manifestations (propositus and relatives with clinical manifestations) and 68 don't present any clinical manifestations (they are healthy or asymptomatic parents or asymptomatic siblings). The genetic samples of these patients were sent to CNAG centre and the quality control was already performed. The genetic results and analysis of these patients were done by IJC.

The obtained variants were mapped in the GRCh38 / hg38 version of the human reference genome. For the prioritization of variants, filters related to pathogenicity and population frequency were applied according to the programs SnpEff v4.1 and Mutation Taster. The allele frequency was evaluated in the population (1000G y ExAC) and in our own data base. Previous descriptions were analysed in general and specific clinical databases of hemolytic anaemia: Human Gene Mutation Database (HGMD) version Professional, ClinVar, Red Cell Membrane Disorder Mutations Database (RDMD), Leiden Open Variation Database- PKLR. In silico predictions of pathogenicity have been performed using Sorting Intolerant from Tolerant (SIFT), PolyPhen-2, Mutation Taster and Mutation Assessor. Reporting of variants have been done following the criteria of the American College of Medical Genetics and Genomics (ACMG). Genetic variants analysis was done by disease category.

A total of 152 patients with Hereditary Haemolytc Anaemia (HHA) were studied with a NGS based panel that contained genes already described in disease causing HHA due to RBC membrane disorders (ANK1, EPB41, EPB42, SLC4A1, SPTA1, SPTB, PIEZO1, KCNN4, RHAG) enzymopathies (ADA, AK1, ALDOA, BPGM, CYB5A, G6PD, GCLC, GPI, GSR, GSS, HK1, NT5C3A, PFKM, PGK1, PKLR, TPI1), hemoglobinopathies (HBA1, HBA2, HBB) and congenital dyserythropoietic anaemias (CDAN1, C15orf41, SEC23B, KLF1, GATA1, KIF23). according to phenotype (clinical and haematological data), 76 patients were diagnosed of membranopathy and oriented as HS (63 patients), HE or congenital piropoikilocytosis (10 patients) and HX (3 patients) The study also included 76 additional patients with inconclusive diagnosis, 25 patients with clinical orientation of mebranopathy and 32 patients without clinical orientation (undiagnosed rare anaemias) . From the patients phenotypically diagnosed of mebranopahy (76) and with clinical orientation of membranopathy (25), the NGS identified the specific mutation in 90 cases, of which 14 had already been reported as disease causing by HGMD. Also, 9 of the 90 cases were already identified in 1000G or ExAC projects with a frequency of less than 0,01. Beta-spectrin, ankyrin and alpha-spectrin were the proteins that gathered most part of the mutations, we identified 33 variants in SPTB, 27 variants in ANK1 and 14 variants in SPTA. 48% (36/74) of the identified variants were missense changes, mostly from SPTB gene (11 variants), while a 38% (28/74) of the variants were nonsense changes or frame shifting mutations, mostly from ANK1 (27 variants) and SPTB (33 variants) (Table 2).

Of special interest, only 2 variants were identified in more than one unrelated family: 1) SPTB c.647G>A, leading to spherocytosis, was identified in 8 patients of 2 unrelated families, and 2) SPTA1 c460_462dupTTG, leading to ellyptocytosis, was identified in 6 patients from 5 different unrelated families.

In 10 families from the 74, two mutations were found as the cause of the RBC membrane disorder, accounting for the 15,63% of the families, thus, presenting an autosomal recessive inheritance pattern.

Finally, with the NGS panel results, the genetic diagnosis of 89% (103/116) of the included patients could be determined and only in 11% (13/116) the mutation was not identified or the variant correlation with disease was not clear. It is worthy to highlight that 10 of the 13 undiagnosed patients had been oriented as unclear membranopathy.

Protein	Gene	Number of patients	Number of families	Number of variants	Previous description HGMD	Identified in 1000G and/ or ExAC
Ankyrin	*ANK1*	31	20	20	1	2
spectrin beta chain	*SPTB*	40	24	24	2	7
spectrin alpha chain	*SPTA1*	35	23	16	3	8
band 3 anion transport protein	*SLC4A1*	14	12	9	5	2
Protein 4.1	*EPB41*	1	1	1	-	-
Protein 4.2	*EPB42*	1	1	1	-	-
Piezo-type mechanosensitive ion channel component 1	*PIEZO1*	5	3	3	3*	

Table 2: Genetic variants from membranopathy patients

According to the results, there is a high genetic heterogeneity in patients with RBC membrane disorders, as almost each family carries a unique mutation that is not observed in any other no related family. The present study reveals the usefulness of NGS panel, which allows the molecular diagnosis of almost the 90%

of the patients and it would avoid misdiagnosis that could lead to splenectomy in contraindicated cases such as in HX. Moreover, the 11% of undiagnosed patients will be analysed through a second NGS gene panel including potential new genes leading to chronic haemolysis and/or sequenced by whole exome sequencing with the aim to identify new disease-causing genes. NGS gene panel is currently being validated for diagnosis porpoises.

1.3 Digital microscopy

The digital microscope developed by UMCU, analyses and classifies certain cell-types by means of the accompanied software. In close collaboration with Albert Schweitzer Hospital (Dordrecht, The Netherlands) UMCU has been running a study to detect and set optimal threshold for many distinct morphological cell types with digital microscopy in hereditary haemolytic anemia.

UMCU has developed a new tool to specifically detect RBC membrane proteins on the surface of the RBC in HS. By using antibodies, we can specifically measure band 3 expression, Rh-factor and RHAG protein and this led to the development of a tool that can detect the underlying genetic cause for HS (Figure 19). This tool can be either used in diagnostics or as a functional assay to validate findings obtained from NGS panels (i.e. classify identified mutations as pathogenic or benign).

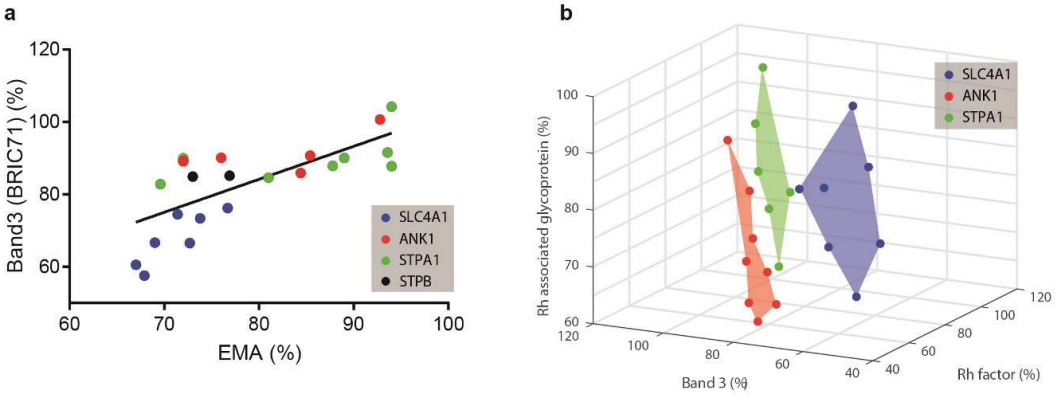
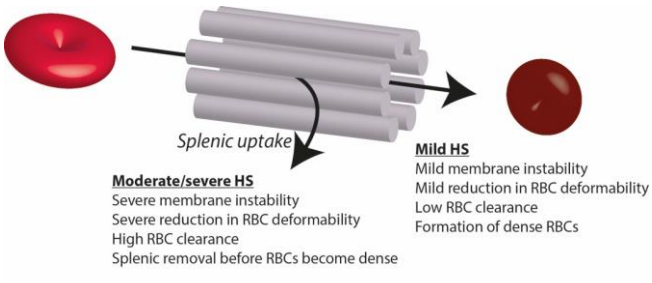


Figure 19: Measurement of band3, Rh-factor and RHAG protein and classification of the underlying genetic defect in HS. This tool is more specific than the EMA-binding test.

In the last period the data analysis has been completed by UMCU and UZH. The most important findings are that density, red cell heterogeneity and deformability can serve as markers of clinical severity in hereditary spherocytosis. Accordingly, the following model is proposed: Mild HS is accompanied with prolonged RBC survival, most likely facilitated by the ability of the HS RBC to pass the spleen. The passage of the spleen results in membrane loss and hence in a decreased surface area and denser RBCs. Moderate/severe HS is characterized by early uptake of the RBCs in the spleen decreasing the RBC life time. This leads to normal RBC densities, most likely because the RBC is cleared before any cell-alterations happen in the spleen.



1.4 Altered cellular ion homeostasis

Blood samples of patients with (HS), HX, beta-thalassemia and sickle cell disease (SCD) were also tested. Main conclusion was that point mutations of single genes (e.g. Gardos channel in patients with HX or band 3, spectrin and ankyrin in patients with HS, result in complex abnormalities in multiple ion transport systems moving Na and K (Na,K-ATPase, NHE and NKCC) and Ca2+.

For selected diseases/samples the following other approaches were performed: Detection of band 4.1a:b ratio, detection of reticulocyte number and the number of cells exposing phosphatidylserine to the outer leaflet (flow cytometry), Ion content (flam photometry), Transport activity of Na,K-ATPase, Na,K,2Cl- and K,Cl-cotransporters and the Gardos channel using $^{86}Rb^{+}$ tracer kinetics assay.

According to the Study Protocol, patients with primary and/or secondary disturbances of ion homeostasis were sent from Utrecht and Spain to all the academic CoMMiTMenT partners of this WP5 in order to characterize the ion homeostasis status. At the UZH the following analysis types were performed: Ca2+ handling detection (fluorescence microscopy, flow cytometry), Morphologlical evaluation (microscopy, forward and side scatter), Separation of cells into fractions according to their density on Percoll density gradient. UZH, USAAR and UMCU tested ion homeostasis characteristics of all the included patients, that were, among others, hereditary spherocytosis patients (mutations in band 3, spectrin and ankyrin), thalassemias and idiopathic cases of RA of unknown molecular origin showing abnormal ektacytometric characteristics. Main activities performed:

- The measured parameters by UZH were: cell morphology, cell Ca^{2+} levels and uptake, the number of NMDA receptor copies per cell, cell density, K^{+} leak, Na^{+}/K^{+} content and the number of hyperchrome cells, reticulocyte count, mean cell haemoglobin concentration, ATP and glucose consumption, GSH:GSSG and met-hemoglobin, filterabiity and mechanic stability, membrane protein cleavage.
- Red blood cells of numerous patients were investigated by USAAR for their Ca^{2+}-content and their electrophysiological parameters. This includes patients from partners within the consortium (see database), but also from 'outside' collaborators.
- UMCU analysed vesicles content, glutathione status and intracellular sodium and potassium of the patients included. Moreover, the consequence of irradiation of RBC concentrates in intracellular potassium and RBC vesiculation is also being studied.

2. Identification and characterization of the molecular players involved in deregulation of RBC ion homeostasis

Two more manuscripts will be produced describing novel approaches to diagnose rare anemias (spherocytosis, channelopaties) in the absence of access to genetic analysis tools and assess disease severity in patients. All this work is performed to formulate the "fingerprints of the disease". These approaches involved quantification of morphological responses of individual RBC to stressors in flow or in static conditions of live cell imaging. A test involving exposure to acid in flow acquired in MeCheM 1 device prototype revealed propensity of individual cells to swelling-induced haemolysis. Analysis of the readouts involved quantification of the "time to response" and "severity of stress required to induce response" – related parameters (device type 1). Static "Ca2+ pulse test" was developed for assessment of Ca2+ uptake and extrusion. Uptake and extrusion of Ca2+ were visualised using the Ca2+-sensitive fluorescent dye fluo-4. Ca2+ was added to the cells suspended in Ca2+-free medium and kinetics of its movement across the membrane monitored over time by means of fluorescence microscopy. Ca2+ uptake and the following

dehydration due to activation of Gardos channels was used as a readout. We also developed a high throughput equivalent of this test involving flow cytometry.

Static test involving measuring of projected areas of each individual cell with the following analysis of the obtained data was shown to give fair prediction of the localisation of mutation causing hereditary spherocytosis. Static equivalent of the flow test in which RBCs were allowed to swell and lyse when coming in contact with acidic gradient (MeCheM1), included administration of a bolus of acid to RBCs in the course of live cell imaging. Such a test revealed the characteristic shape changes reflecting partial dissociation of cytoskeletal network from transmembrane protein complexes. Further studies to characterize the power and limitations of each of these tests are currently on-going.

Regarding collaboration between UZH and Epigem, the initial MeCheM devices were supplied to UZH and promising results obtained. Epigem's MeCheM technology developed in WP2 and WP3 was applied initially with UZH for high content screening, clinical diagnosis, identification of pharmacological targets and treatment of RBC channelopathies / channel related disorders. Epigem work in period M47-54 accelerated the development of "MeCheM" RBC analytical test methods combining low cost imaging optics with microfluidics. The usefulness of MeCheM in relation to WP5 relates with the activity of particularly on identification of molecular players. Epigem's technology is generating results that can differentiate the different types of rare anaemias.

3. Pharmacological screens, to identify drugs with potential effects applicable for selected RAs treatment

Proof of principle single center placebo-free phase II for supportive use of an ion channel blocker, antagonist of NMDA receptor memantine, for treatment of patients with symptomatic sickle cell disease, was finalized on 31.03.2017 by the University Hospital Zurich (NCT02615847). Preliminary biological data were presented at the 21st ERCS Red Cell Club Meeting in Heidelberg in April-May 2017. Within the trial patients with 5+ years of observation history were exposed to treatment with incremental daily doses of Memantin Mepha from 0 to 20 mg a day over the first month (phase I) and maintained the dose at 20 mg/day for the next 10 months (phase II). Following one further month of down-dosing from 20 to 0 mg memantine a day (phase III) patients were observed for the next 2 months of a challenge phase being off the drug (IV).

Selected findings form the trial generated by the UZH team on the biological effects of memantine on red blood cells of patients on the trial are presented in Figures 20-22. In brief, treatment with memantine resulted in decrease in production of new red blood cells as follows from reduction in reticulocyte counts. At the same time the number of circulating red cells was not decreasing suggesting improved longevity of the circulating red blood cells (Fig 20). Hemolytic activity markers revealed advancements in red blood cell stability (Fig 21). Memantine supported re-hydration for red blood cells. Decrease in MCHC and abundance of hyperchrome and dense cells suppresses haemoglobin S polymerisation (Fig 22). In line with that, reduction in number of circulating terminally sickle red blood cells in peripheral blood.

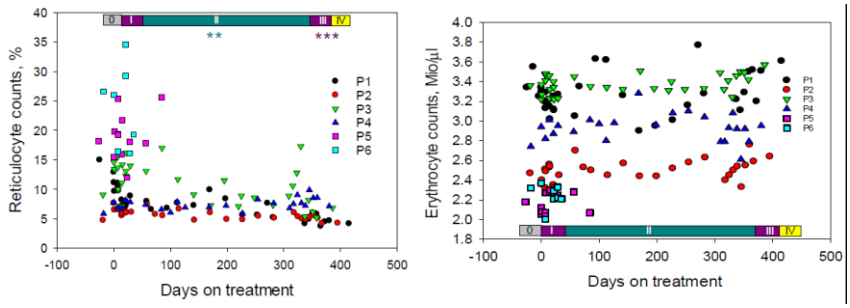


Figure 20: Alterations in reticulocyte and erythrocyte counts during up-dosing (I), treatment (II), down-dosing (III) and challenge (IV) phases of the trial (see the color-coded bar). *** and ** stand for p<0.001 or p<0.01 compared to the values before the onset of treatment (phase 0).

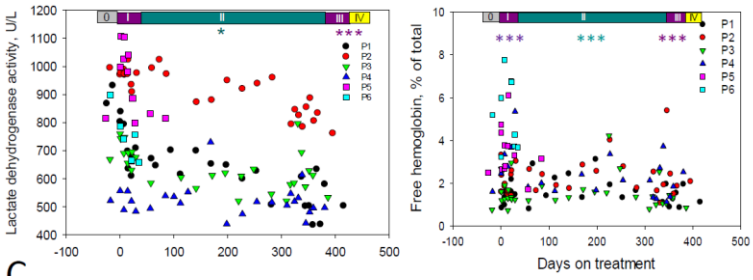


Figure 21: Decrease in hemolytic activity assessed as plasma lactate dehydrogenase activity and mechanic stability of red blood cells detected as free haemoglobin released after filtration through the cellulose column)

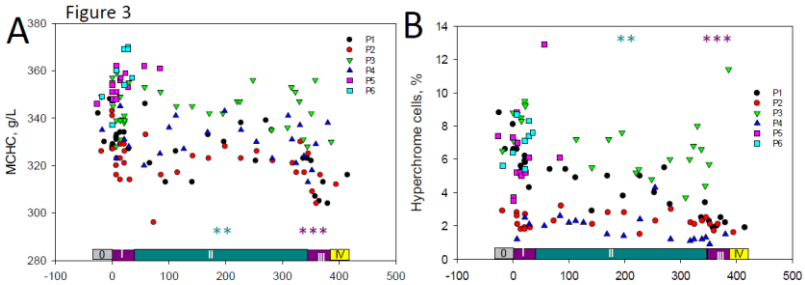


Figure 22: Reduction in mean corpuscular haemoglobin concentration (MCHC) and decrease in abundance in hyperchrome cells in patients during the trial.

3.5. Work Package 6 – Validation of the combined imaging technologies

Main objectives of WP 6

O6.1: Set up and tuning of a fully functional integrated device (µCOSMOS).

O6.2: Measurement of the specified parameters (WP 5) with the µCOSMOS device as a proof of principle.

O6.3: Determination of personalized doses for the drug memantine for a cohort of sickle cell disease patients.

Activities and results of WP 6

SICM has been optimised to achieve successful imaging on Red blood cells (RBCs) with SICM. Optimum scan parameters have also been found for RBC scans. Living RBCs from RA patients and fixed deoxygenated RBCs have been scanned with high resolution in SICM (see WP4 description).

Epigem has developed two types of prototype instrument, fabricated large numbers of devices for testing and iterated design improvements of the instrument pumping system and optoelectronics, the disposable microfluidic test components and the process control and imaging software and made multiple software and standard operating procedure improvements in response to user feedback. MeCheM 1 *flow analyser* is a microfluidic system that counts flowing red blood cells and provides a characteristic curve as the diagnostic biomarker. MeCheM 1 is being used to monitor the effect of treatment in an ongoing memantine drug trial that commenced in Israel in June 2018 led by UZH.

Three Mark 1 MeCheM prototypes were built for validation trials at EMEK Afula, UZH Zurich and Sanquin, Amsterdam. The feedback from these trials has been embedded in improvements:

1. Control and analysis software e.g. measurement point standard deviation reduction.
2. Operation procedure improvements, mainly as a result of extensive testing by Epigem and feedback derived via remote maintenance support of the user at EMEK.
3. Design and build of a more robust mark 2 folded metal prototype with improved access to key components for maintenance.

An important achievement has been the construction of a new optofluidic benchtop prototype which we are calling MeCheM 2 *drop analyser*. MeCheM 2 is a simple drop (microlitres) on a surface test. Within a stationary drop red blood cells can be imaged at higher magnification and single cell tests performed, including surface texture measurements. The advance has been that improvements in the optical system now provide enough contrast for automated cell projected area measurement.

The main differences between MeCheM 1 and 2 is the number of cells that can be conveniently tested in a few minutes and the effect of flow. We also envisage that a portable version of MeCheM 2 will be possible, but to keep the cost down, may not be as versatile as MeCheM 1, which in principle could have the drop tests integrated into it as an option.

The main USP (unique selling point) we anticipate is being able to follow changes in specific cells of interest, within a population of cells, determined by the field of view or multiple fields for statistical benefits.

MeChem 1 flow analyser

Internal work within Epigem on MeCheM 1 has focused on understanding / calibrating the curves, which are the test biomarkers, and the factors responsible for variation. These include gender, age, venous (arm vein) vs capillary (finger prick) sampling, RA type as well as process operating procedures, device design and device, fabrication related dimensional tolerances. Parallel work on the patient dependent parameters has been done by UZH.

MeCheM 2 *drop analyser - shape* analysis by projected area test

MeCheM 2, a static diluted blood drop test has now also reached the "boxed" prototype stage and is able to perform a project area shape analysis measurement. Epigem addressed the contrast problem and now, with both significantly enhanced image contrast and magnification, can automatically analyse and output projected area histograms, or spectra, using Open Source software.

The next trials of both instruments are being planned. MeCheM 1 and 2 are both designed for bench top use in a laboratory or clinic. Several additional tests are expected to be included as options in response to end user needs. Fluorescence imaging is next. Feedback is rapidly increasing now a useful product can be seen and tested and comparisons made with alternative approaches. Unique selling points are becoming clearer and a more detailed market segmentation and value assessment can commence.

Detailed patent data are available for both prototypes; however, they cannot be shared due to IP reasons at this point in time.

4 Description of the potential impact

The consortium has been designed to provide a dynamic synergy between the SMEs and the academic and clinical partners. This collaboration allowed for direct access and knowledge transfer without the investment of outside knowledge and expertise. Additional value had been provided through well-established connections of consortium members to the anaemia medical research and clinical diagnostics communities as well as to hospitals and patients. The integration of the commercial SMEs with the academic research organisations provided a culture of innovation within the consortium that will survive beyond the duration of the project.

The impact considerations of **CoMMiTMenT** can be divided into three subsections: (i) medical impact on the diagnosis, treatment, and personalised medication to treat RA; (ii) scientific/technological impact on the development of combined microscopy methodologies; and (iii) economic impact. These are addressed in detail below:

(i) Medical impact on the diagnosis, treatment, and personalised medication of RA

Newly emerging genomics and proteomics technologies are generating data on a scale that is unprecedented in biomedical research and requires complementation by functional data on the cellular and/or molecular level. **CoMMiTMenT** filled in this gap.

RA are a group of rare diseases, of which the most significant clinical manifestation is anaemia. In Europe, the group of patients with RA is very heterogeneous. Corpuscular defects of RBCs are one of the three major causes of anaemia, with the other two including disorders of RBC production and disorders of erythroid maturation and ineffective erythropoiesis. Nearly all cases of RA are congenital or hereditary. An overview of RA, their prevalence and clinical management are summarised in Table 2.

Due to their rarity, the precise frequency of the majority of the diseases that will be investigated is unknown. As mentioned above, their occurrence is less than 5 per 10,000 individuals in the European population. A subset of these disorders shows a prevalence of less than 1 per 50,000 individuals in Europe, depending on the geographical area. **CoMMiTMenT** assumed and required the inclusion of a significant number of patients with each of the following rare diseases to draw reliable (statistical) conclusions: haemoglobinopathies (at least 200, primarily thalassemias and SCD), enzymopathies (at least 60 cases, primarily PK and PFK deficiency, and other enzyme defects associated with neurological disease), and membrane defects (at least 100 cases, primarily HS and hereditary stomatocytoses). Moreover, at least 100 cases of RAs without known aetiology will be required (*i.e.*, undiagnosed RA). It is worth mentioning that approximately 20% of RAs that are seen in clinical practice remain undiagnosed due to the lack of appropriate diagnostic tools for these disorders.

In combination with the fact that there is generally little awareness of the existence of many of these disorders, a limited but significant number of cases has been recruited on a national level. Moreover, as rare specific mutations generally affect only a limited number of families, combined effort of different European experts has been critical for achieving a broad geographical coverage. Lastly, given that it has been clearly demonstrated that the expertise in RAs in the individual European countries is variable, it has become evident that cross-border cooperation is by far the best way to recruit the highest expertise and a sufficient number of patients. The members of ENERCA have been extremely helpful for the recruitment of patients and for increasing the expertise of **CoMMiTMenT** participants in the types of diagnosis and treatment of RA that have been included in this project.

One of the major advantages of the developed technologies has been the generation of new intellectual property that will protect the novel diagnostic techniques and diagnostic devices for the diagnosis of RBC diseases. This technology will be of potential interest for both hospital and *in vitro* diagnostic companies and pharmaceutical industry.

Table 3: Overview of RA, their prevalence and current clinical management.

Type of Anaemia	Frequency in the population	Number of tests undertaken	Cost of research or to health services	Mortality rate	Cure today (yes or no)	Likelihood of cure
Sickle Cell Disease	Europe: Variable depending on the country < 1/5,000	120-500 per year	High due to frequent hospitalizations and blood transfusions	8% in early childhood, and < 1% if early diagnosis by newborn screening	No	Hydroxyurea/ Allogenic stem cell transplantation
Thalassaemia	Mediterranean about 1.5-2%	600-1500 per year (minor and intermediate) About 100 per year (major or Cooley anaemia)	High only thalassaemia major due to transfusion requirement and iron chelation	About 2% in severe forms of thalassemia major	No	Allogenic stem cell transplantation /gene therapy
PFK deficiency and other rare RBC enzymopathies	Very rare: < 1/ 100,000	Variable: 50-80 per year	Unknown, (too rare)	Variable: TPI deficiency: 80% before the age of 20	No	Allogenic stem cell transplantation
Hereditary spherocytosis	1/5,000	100-150 per year	Depending on the severity of the anaemia. In general moderate	< 0.05%. In general after splenic-tomy only	No	Splenectomy
Hereditary stomatocytosis (xerocytosis/ hydrocytosis)	< 1/50,000	10-50 per year	Depending on the severity of the anaemia.	< 0.01%.	No	Unknown

Interestingly, protein malfunctions in the RBCs of patients with enzymopathies (*e.g.*, deficiencies of PK and PFK, and perhaps others), membranopathies (HS and hereditary stomatocytosis, such as xerocytosis,

overhydrated hydrocytosis and cryohydrocytosis) and undiagnosed RA are directly or indirectly associated with abnormalities in water and ion homeostasis and extensive calcium leakage. Unfortunately, despite the great technological advances that have been made in the past 20 years, the diagnostic tools that are available for RA are poor and, as mentioned above, an important group of these patients remain undiagnosed or even misdiagnosed.

CoMMiTMenT contributed to a better understanding of the complex pathophysiology of these rare RBC disorders that are associated with altered ion homeostasis, either as a primary or a secondary cause. Such knowledge is incomplete or wholly missing due to the lack of high-throughput screening tools. The project resulted in the generation of a substantial body of knowledge with respect to the involvement of ion homeostasis in all forms of RA, with or without a known mechanism. This project also identified involved ion transporters and suggested possible pharmacological interventions especially for sickle cell disease (memantine) and for the Gardos channelopathy (senicapoc).

Accordingly, **CoMMiTMenT** developed a state-of-art approach to achieve the following aims/outcomes: (i) the ability to accurately diagnose these disorders, e.g. a high-throughput approach to identify altered Piezo1 activity in hereditary xerocytosis patients; (ii) the identification of the ion transporters that produce the aberrant ion / water balance, e.g. in the Gardos channelopathy; and (iii) monitoring of the effect and efficiency of pharmacological interventions, e.g. clinical trials using different doses of memantine to treat sickle cell disease patients.

Many of the current analytical procedures used for the diagnosis of these rare diseases are, in general, cumbersome and highly time-consuming. Furthermore, the high level of expertise and technical skills that they require, as well as the required working conditions, may not always be available, even in laboratories that are specialised in the advanced diagnosis of haematological disorders. Therefore, in Europe only very few highly specialised laboratories are performing some, but not all of the analytical procedures that are necessary for the diagnosis and follow-up of the RA that have been examined in **CoMMiTMenT**. Moreover, RA due to specific RBC transmembrane ion transport abnormalities are so rare that only a small number of laboratories in Europe have access to specialists who are able to assess the function of individual ion transporters (*e.g.,* ion channels, non-electrogenic symporters or antiporters or ion pumps). Since these highly specialized procedures are not automated, they lack standardisation and national or local External Quality Assessment Schemes (EQAS), which are not implemented due to its excessive cost. The European Network for Rare and Congenital Anaemias (ENERCA), supported by EC DG-SANCO, has ameliorated this situation by facilitating the identification of experts and expert centres dealing with RAs and enabling an efficient method for exchange information and expertise (www.enerca.org). Accordingly, the active participation of ENERCA experts in this project provided patients, expertise and access to patients from several health centres throughout Europe.

There is no doubt that this project has rendered new scientific insights and valuable information regarding the pathophysiology of RA. This knowledge has been and will be applied to the development of novel diagnostic tools (*e.g.*, MeCheM, SICM high throughput patch-clamp) and/or be used for the development of novel therapeutic strategies. In fact, patients will benefit from the development of newly improved diagnostic tools, a faster diagnosis time and several pioneering treatment options. As a whole, these advances will lead to a simplification of diagnostic testing and to a reduction of hospitalisation requirements. Moreover, the new diagnostic tools may be used to set up and monitor the progress/outcome of personalised treatments and improve patient's quality of life. In summary, **CoMMiTMenT** results will lead to patient's clinical care improvement in all aspects of diagnosis and follow-up, as well as to the medical decisions that are involved in treatments, such as supportive treatment (blood

transfusion) and splenectomy, the outcome of which is generally difficult to predict. The project has allowed for these decisions to be made in a far more sophisticated way and contributed to the development of novel therapeutic options and eventually provided MeCheM, a new tool for the establishment of personalised medication. The possibility of correcting the intrinsic abnormalities of RBCs causing sickle cell disease will improve the quality of life by decreasing concomitant complications such as vaso-occlusive pain crisis (publication of clinical trial in preparation).

The transition of personalised medicine into clinical practice has been repeatedly argued as being necessary for sickle cell disease (SCD), which is currently tested for memantine in a clinical trial in Afula (Israel). A further therapeutic strategy for SCD (in addition to memantine) is based on reducing the cellular concentration of HbS through the prevention of RBC dehydration due to the activation of Ca^{2+}-sensitive K^{+} channels. Further consequences of high intracellular Ca^{2+} levels include oxidative stress and cleavage of cytoskleletal proteins by Ca^{2+}-sensitive proteases making cell prone to lysis and premature clearance.

The outcome of **CoMMiTMenT** can be summarised under three major points:

Socio-economical: By increasing the **diagnostic capacity** and the **efficacy of medication**, the **quality of life** and the active productive life of patients with chronic hereditary anaemia can be improved, thereby **reducing hospitalisation cost**s

Clinical perspectives: By improving the quality of diagnosis, the diagnostic process will be expedited. This achievement alone reduces hospitalisation, medication and labour costs. Improving the application of a correct and personalised treatment and reducing medication costs by tailored interventions and achieving a predictable outcome of personalised therapy are themselves major advancements.

Clinical research: By unifying the efforts of all known European medical scientists, the clinical care this is required for the very few cases of largely unattended patients with RA who are currently present in a given hospital, centre or European country will significantly decrease.

The novel treatment modalities explored in **CoMMiTMenT** offered possibilities for intellectual property on new drugs and treatment strategies of these disorders, which are of interest for pharmaceutical companies.

Apart from results that offer commercialisation opportunities, the results of this project allowed for a new classification of rare RBC disorders as channelopathies. The increased understanding of the pathophysiology of these disorders may help the field understand the primary and secondary consequences of the disease and provide opportunities for improved patient care.

(ii) Scientific/technological impact on the development of combined microscopy methodology (µCOSMOS)

The combination of two imaging technologies (MeCheM and SICM), is a novel approach that provides tremendous potential to solve a variety of scientific questions but which was initially used to identify novel targets to treat RA. This project combined conventional and novel methodologies to measure parameters that have never been successfully measured (e.g. mechanochemical probing of RBCs and high throughput patch-clamp). This will be accomplished in the context of structural and functional RBC disorders that are characterised by increased RBC fragility and shear stress-intolerance. This combination of technologies improved the understanding of ion homeostasis abnormalities and their relationship/s with the intrinsic RBCs defects in several groups of patients with RA.

The scientific use is not limited to the diagnosis and therapeutic interventions of rare diseases but extends into numerous biomedical applications, including basic research in neuroscience, cardiovascular and oncology. The IP generated within **CoMMiTMenT** will open new application fields for the SMEs involved. This holds true for the microscopy approaches as well as for the microfluidics and image analyses / processing techniques. A great advantage of **CoMMiTMenT** is that it will allow for the use of the novel technology to diagnose RBC channelopathies in patients who suffer from the same RA.

Although CoMMiTMenT was focused on RA, the developed methodology can also be readily used to explore and eventually treat other rare diseases that are associated with channelopathies. This holds true for blood cells other than RBCs (*i.e.*, leukocytes and platelets) as well as for all other cells that can be obtained by biopsy and properly dissociated.

Furthermore, there are numerous potential applications of the imaging technologies beyond rare diseases. For instance, there are further RBC-related biomedical challenges. These include the establishment of personalised medication for dialysis patients who suffer from anaemia, as well as quality control in transfusion medicine. The latter point is a particularly important issue, as indicated by the serious hazards that are faced in RBC transfusion.[1,2]

[1] Luten, M. *et al.* Survival of red blood cells after transfusion: a comparison between red cells concentrates of different storage periods. *Transfusion* **48**, 1478–1485 (2008).

[2] *Annual SHOT report 2011*. (Serious Hazards of Transfusion (SHOT) Steering Group, 2012).

5 Address of project public website and relevant contact details

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