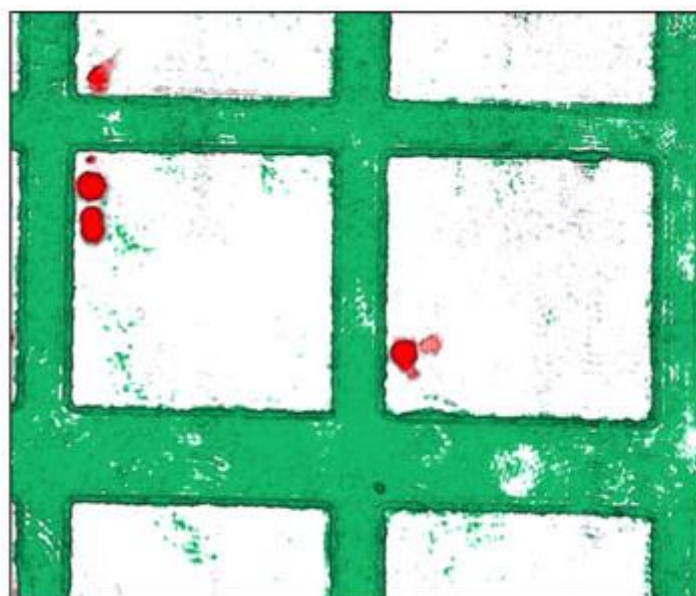


Application Note

Quantitative Long-Term Monitoring Of Non-Adherent Cells By Digital Holographic Microscopy



By Cosette J and Stockholm D

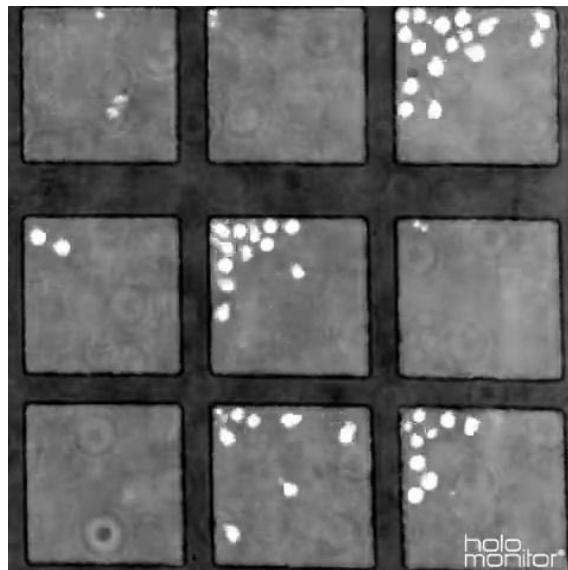
EPHE-PSL University, Paris, France

Genethon, Evry, France

Introduction

Time-lapse microscopy is the method of choice for studying the dynamics of cell processes. Non-adherent cells, such as hematopoietic stem cells or lymphocytes, provide challenges for live imaging as they migrate rapidly and are likely to leave the field of view. Special coatings and staining of any type, including living cell labelling, could affect the physiological relevance of the study by somehow activating cells in an uncontrolled way (Gilner *et al.*, 2007). As a way to circumvent these problems, a methodology was developed by Daniel Day (Day *et al.*, 2009) that relies on the use of biocompatible uncoated silicone microgrids containing 60 μm walls. These walls prevent cells from escaping the field of view without restricting cell motility and cell to-cell interactions.

In this work we show that the dynamic changes of cord-blood derived CD34+ cells in response to cytokine stimulation can be successfully studied, in a label-free way, using digital holographic microscopy. This approach can be easily applied to any type of non-adherent cell monitoring.



A digital holographic microscopy time-lapse of human CD34+ cells in a microgrid, captured by HoloMonitor®.

Materials and Methods

Cell Isolation and Culture

Human umbilical cord blood (UCB) was collected from placentas and/or umbilical cords obtained from Saint Louis Hospital, France in accordance with international ethical principles and French national law (bioethics law n82011–814) under declaration N8DC-201- 1655 to the French Ministry of Research and Higher Studies. Human CD34+ cells were isolated using autoMACS pro (Miltenyi Biotec, France) by immunomagnetic selection based on human CD34 expression within the mononuclear cell fraction of UCB samples. Cells were cryopreserved in Cryostor (StemCell, France) and stored in liquid nitrogen.

Immediately after thawing, cells were put in an activation medium, whose base is XVIVO medium (Lonza) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin (Gibco, Thermo Scientific), that contains activation cytokines: 50 ng/ml h-FLT3L, 25 ng/ml h-SCF, 25 ng/ml h-TPO, and 10 ng/ml h-IL3 (Miltenyi Biotec, Paris, France). Cells were cultured and imaged in this medium for the whole study in a humidified 5% CO₂ atmosphere at a temperature of 37°C.

Microgrid Preparation

A microgrid array (Microsurfaces, Australia) made of Polydimethylsiloxane (PDMS – biocompatible silicone) containing 1,024 micro-wells (125 μm width, 60 μm depth) was placed in an uncoated 35 mm dish (Ibidi, Germany) with a polymer coverslip bottom (Figure 1). The transfer is operated by peeling the microgrid from its support with a pair of tweezers and depositing it directly on the glass. A volume of 200 μL of EtOH 100% is added onto the microgrid for 5 min to sterilize it and minimize contamination risks during time-lapse imaging. EtOH is then pipetted out and the microgrid is left to be dried 30 min under a hood. The whole dish was then filled with cell culture medium. Five thousand cells were loaded above the microgrid array and left 10 min for sedimentation. According to Poisson's law, about 30% of microwells are gifted a single cell.

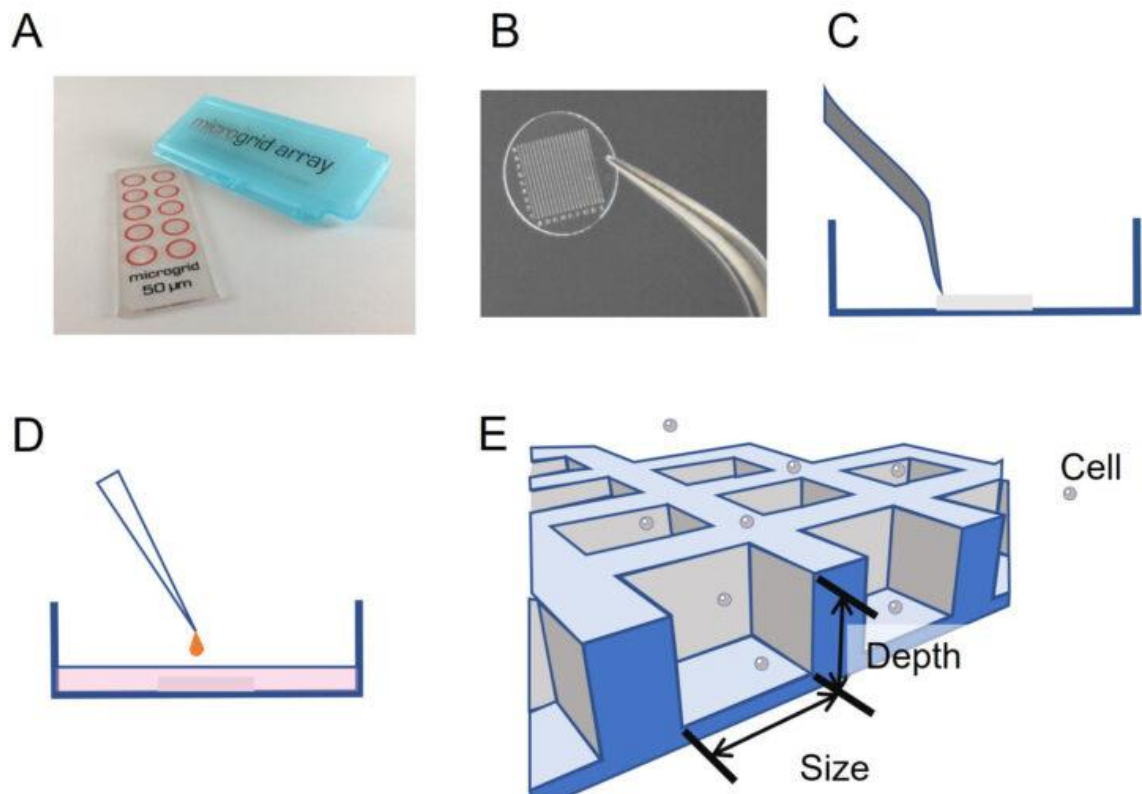


Figure 1. Method to prepare the microgrid in the 35 mm dishes. Microgrids (A) are peeled off a special slide with a pair of tweezers (B) and are placed inside 35 mm dishes (C). The dish is filled with medium and cells are dropped from above (D). Cell sedimentation results in the isolation of individual cells in the 125 μm -sized and 60 μm depth (E), according to Poisson's law.

Time-lapse Microscopy

Time-lapse acquisitions were performed with HoloMonitor® M4 – placed inside an incubator (5% CO₂ – 37°C). Twenty field positions were recorded covering 4 microwells each. Images were acquired every 10 minutes for 4 days using a 20X objective. Images were processed with HoloMonitor® App Suite software. Data were also exported, and the cell parameters eccentricity and motility were processed with R (R Core Team, 2020) for graphics purposes. The parameter eccentricity describes how much the cell deviates from being a circle. A value of 0 corresponds to a circle and the more elongated the cell is the higher the eccentricity value becomes, approaching 1. The motility parameter gives the actual distance travelled by the cell between the starting point and the endpoint of the cell path.

Results

Morphology Determination

Previous studies have demonstrated that there is a connection between cell morphology and the differentiation potential of CD34+ cells. Two major morphological forms have been described in the CD34+ cord blood cell fraction. Polarized cells with an amoeboid shape are capable of active motion by vigorous shape change and generally possess at one end a large protrusion called uropod. These cells have been found to retain primitive self-renewing and stem cell functions (Gorgens *et al.*, 2014). The second morphological type is round. These cells have been considered as already engaged in differentiation (Wagner *et al.*, 2004).

Images obtained with HoloMonitor® M4 revealed that the two cell morphologies previously described can be easily observed. After recovering from the stress of isolation and manipulation, founder cells (i.e. cells that were initially put in the microgrid) acquired polarized morphologies within a few hours, developing uropods and starting a phase of active motion (Figure 2).

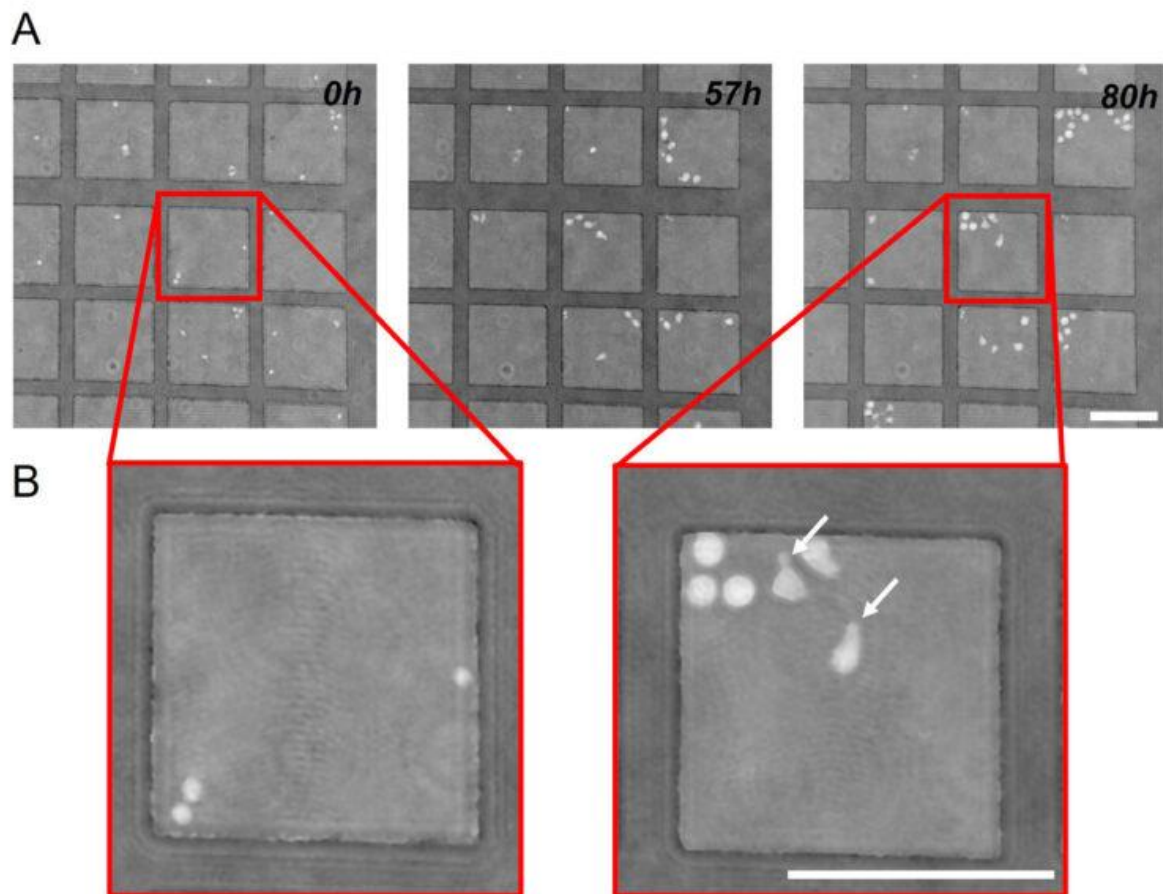


Figure 2. Panel A: Field of view showing nine complete microwells followed during 96h. Panel B: Left image shows an enlarged view of the central microwell. Cells at time 0h are small and round. The right image is the view of the same enlarged microwell at time 80h. Three round cells are located on the top left of the microwell and three polarized cells are visible on the right. Visible uropods of polarized cells are shown with white arrows. Scale bar: 100 μ m.

Motility and Eccentricity Quantification

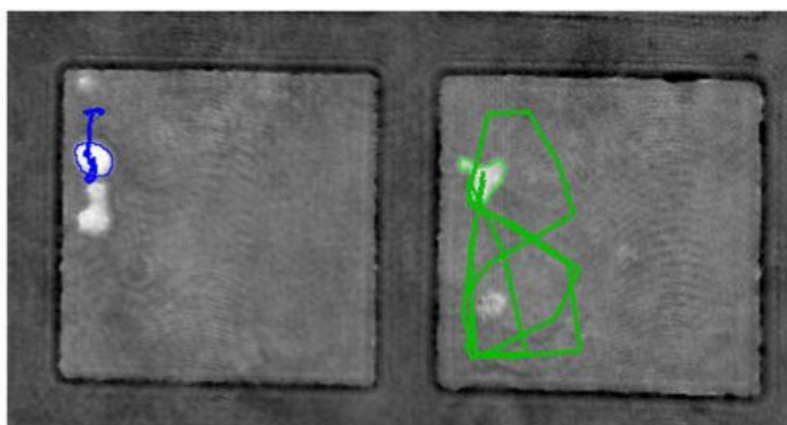
To illustrate the capacity of the system to quantify both the shape and the motility of cells, we explored two parameters that are easily computed with the cell tracking device. We monitored two types of cell behaviour which seem to be correlated with the type of cell shape. Polarized cells tend to have strong motility while round cells exhibit low motility. Quantification of the eccentricity is a good means to classify a cell into one of the two categories (polarize vs round). We have shown an example of differences in cell behaviour connected to the two categories during monitoring of four hours with a focus on two particular cells (Figure 3). The round cell with an average eccentricity of 0.36 ± 0.15 shows low motility which does not exceed 100 μm with a track restricted to a very small area in the upper corner of the microwell. On the other hand, the polarized cell in the neighbouring microwell displays a wide track, covering almost 40% of the well surface area with motility climbing up to 600 μm , while its eccentricity has an average of 0.72 ± 0.12 (Figure 3C).

Growth of the Cells During the First 96 Hours

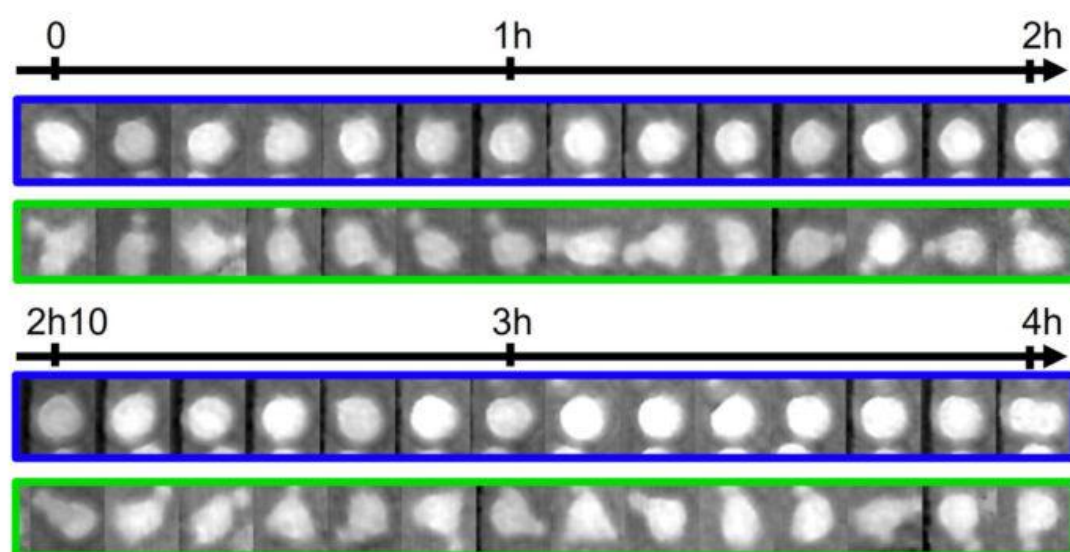
Cell dry mass can be determined by quantitative phase imaging (Barer 1952 and Aknoun *et al.*, 2015). The refractive index of solution was shown to be the sum of the solvent refractive index and an increment proportional to the solute mass density. We applied this technique of measurement to distinguish the cell growth evolution of the first generation compared to the second generation (Figure 4). This approach has the advantage to avoid any modification of the sample.

The results show a clear difference in the aging between the first generation compared to the second generation for which the age of the cells is much shorter than previously described (Moussy *et al.*, 2017 and Cosette *et al.*, 2017). Furthermore, the HoloMonitor® M4 system enables us to quantify the cell dry mass based on optical volume for a significant number of cells, demonstrating that the shorter time for a division is associated with a smaller differential of growth compared to the first generation (Figure 4 C).

A



B



C

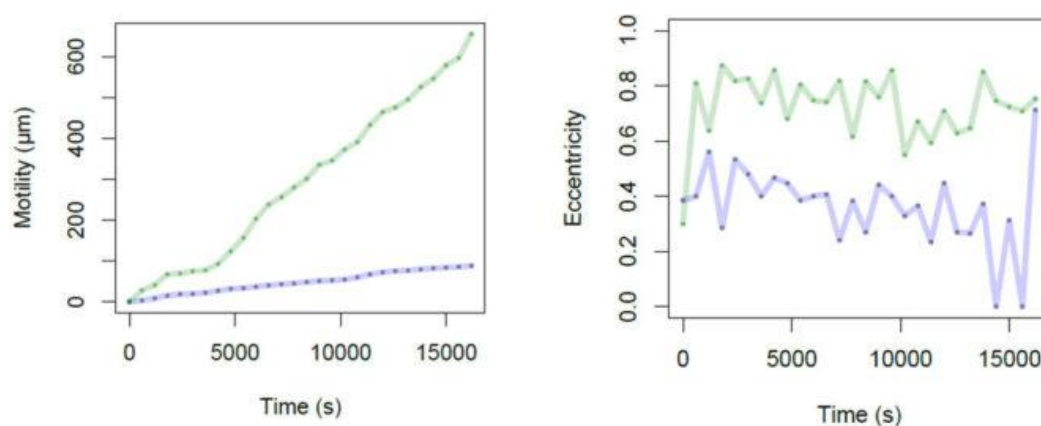
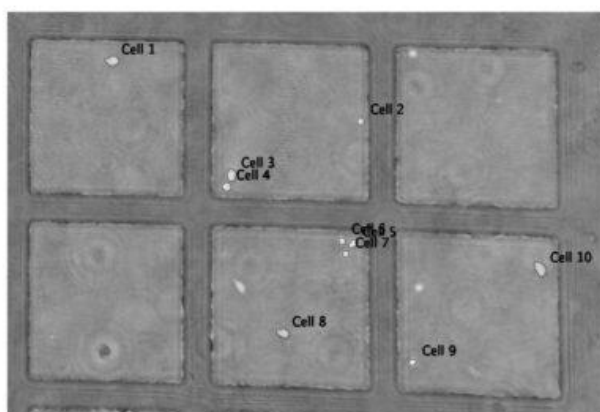
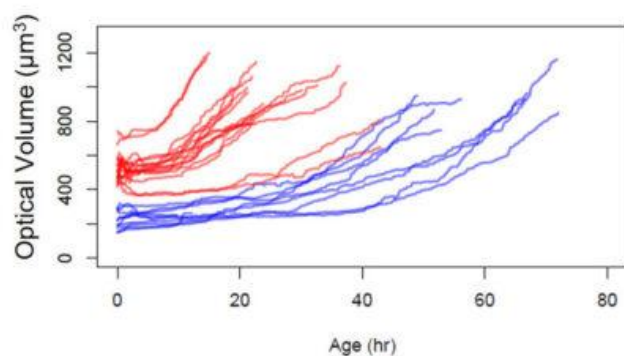


Figure 3. Panel A shows a crop of the image view showing the two cells that were monitored for 4 hours. The microwell on the left contained two cells, one of which had a round shape with small motility. Its cell track is shown in blue. The microwell on the right contains a polarized cell and its track is shown in green. Panel B shows individual images of these cells during the time of the study. Panel C presents the quantification of motility and eccentricity of these two cells (polarized cell in green and round cell in blue).

A



B



C

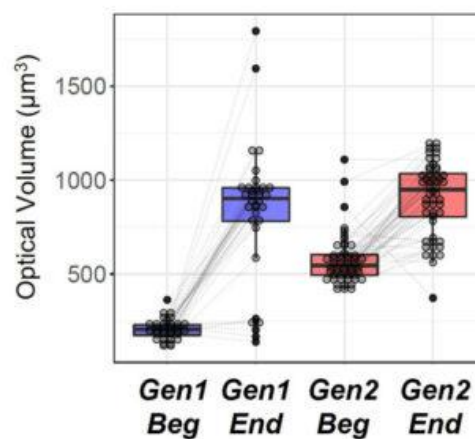


Figure 4. Dynamics of the optical volume of 10 cells (numbered in panel A) are shown in panel B. Monitoring of cells from the first generation is shown in blue whereas cells from the second generation are shown in red. Panel C shows the statistics of the optical volume for a total of 34 cells which are first-generation cells from the beginning of the study (Gen1 Beg), and just before their cell division or their cell death (Gen1 End), as well as their cell daughters at age 0 (Gen2 Beg) and just before their cell division or their cell death (Gen2 End). The few cells without any significant growth correspond to dying cells.

Discussion

We show in this report the feasibility to follow, at the single-cell level, cell morphology, cell motility, and cell growth of non-adherent cells during several days thanks to the use of a microgrid array combined with a quantitative phase imaging system.

Holographic microscopy is a label-free approach, therefore avoiding any modification of the sample or any labelling that could change the fate of the cell, especially hematopoietic CD34+ stem cells (Gilner *et al.*, 2007).

The combination of the microgrid array and quantitative phase imaging overcomes the cell motility challenge of time-lapse microscopy of non-adherent cells, which is their very ability to get out of the field of view, or out of focus.

Following changing cell morphology characteristics requires a strong and precise segmentation, which usually remains a challenge for phase-contrast images. On the other hand, digital holographic images contain more information, then enabling a very satisfactory and greatly improved cell segmentation when associated with HoloMonitor® software algorithms.

One of the strong benefits of using the HoloMonitor® M4 compared to standard methods is the ease of quantification of critical parameters like the optical volume which can be directly related to cell growth. It also offers the possibility to capture rare events in subpopulations of cells (e.g. dying cells). The overall advantages of this approach are a substantial gain of time due to the automatic image analysis requiring only a few manual corrections, the accurate estimation of cell dry mass, and the possibility to simultaneously study different conditions.

We show in this report that quantitative phase imaging is compatible with PDMS, paving the way for single-cell-on-chip analysis, for instance on microfluidic chips. Furthermore, the ability we show to monitor motility and trajectory patterns of cells could be of great interest to many fundamental research fields or in the study of diseases in which cell motility plays a key role such as metastatic cancer disease.

References

- Aknoun S, Savatier J, Bon P, Galland F, Abdeladim L, Wattellier B, Monneret S. Living cell dry mass measurement using quantitative phase imaging with quadriwave lateral shearing interferometry: an accuracy and sensitivity discussion. *J Biomed Opt.* 2015; 20(12):126009.
- Barer R. Interference Microscopy and Mass Determination. *Nature* 169, 366–367 (1952). doi: 10.1038/169366b0.
- Cosette J, Moussy A, Paldi A, Stockholm D. Combination of imaging flow cytometry and time-lapse microscopy for the study of label-free morphology dynamics of hematopoietic cells. *Cytometry A.* 2017 Mar; 91(3):254-260.
- Day D, Pham K, Ludford-Menting MJ, Oliaro J, Izon D, Russell SM, Gu M. A method for prolonged imaging of motile lymphocytes. *Immunol Cell Biol.* 2009 Feb; 87(2):154-8. doi: 10.1038/icb.2008.79.
- Gilner JB, Walton WG, Gush K, Kirby SL. Antibodies to stem cell marker antigens reduce engraftment of hematopoietic stem cells. *Stem Cells.* 2007; 25:279–288.
- Gorgens A, Ludwig AK, Mollmann M, Krawczyk A, Durig J, Hanenberg H, *et al.*, Multipotent hematopoietic progenitors divide asymmetrically to create progenitors of the lymphomyeloid and erythromyeloid lineages. *Stem cell reports.* 2014; 3(6):1058– 72.
- Moussy A, Cosette J, Parmentier R, da Silva C, Corre G, Richard A, Gandrillon O, Stockholm D, Paldi A. Integrated time-lapse and single-cell transcription studies highlight the variable and dynamic nature of human hematopoietic cell fate commitment. *PLoS Biol.* 2017 Jul 27; 15(7):e2001867.
- R Core Team (2020). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <https://www.R-project.org/>.
- Wagner W, Ansorge A, Wirkner U, Eckstein V, Schwager C, Blake J, *et al.*, Molecular evidence for stem cell function of the slow-dividing fraction among human hematopoietic progenitor cells by genome-wide analysis. *Blood.* 2004; 104(3):675–86.