Invited Paper

Optical quantification of forces at play during stem cell differentiation

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ABSTRACT

A cell is in constant interaction with its environment, it responds to external mechanical, chemical and biological signals. The response to these signals can be of various nature, for instance intra-cellular mechanical re-arrangements, cell-cell interactions, or cellular reinforcements. Optical methods are quite attractive for investigating the mechanics inside living cells as, e.g., optical traps are amongst the only nanotools that can reach and manipulate, measure forces, inside a living cell. In the recent years it has become increasingly evident that not only biochemical and biomolecular cues, but also that mechanical ones, play an important roles in stem cell differentiation. The first evidence for the importance of mechanical cues emerged from studies showing that substrate stiffness had an impact on stem cell differentiation. Recently, techniques such as optical tweezers and stretchers have been applied to stem cells, producing new insights into the role of mechanics in regulating renewal and differentiation. Here, we describe how optical tweezers and optical stretchers can bee applied as a tool to investigate stem cell mechanics and some of the recent results to come out of this work.

Keywords: Stem cells, differentiation, mechanical cues, optical forces, optical tweezers, optical stretcher, microrheology

1. INTRODUCTION

A stem cell is a special cell endowed with the capacity to differentiate into a range of specialized cell types while retaining the ability to produce progeny that are equally potent, so called self-renewal. For this reason, stem cells are regarded the 'holy grail' of regenerative medicine, and they can be thought of as the body's reserve system for spare parts. In essence, there are two basic classifications of stem cells. In embryonic development, progenitor cells can exist for several cell divisions and these can be expanded *in vitro* to make stem cell lines such as embryonic stem cells (ESCs). There are also stem cells that reside in a tissue during adult homostasis, adult stem cells, that provide for a new source of specialized cells for different organ systems such as the gut, blood or skin.

Based on their capacity to differentiate into different cell types, stem cell potency can be categorized. *In vitro* stem cells, that can generate all cells of the future organisim, including all the extra-embryonic structures such as the placenta, are totipotent.[1] While, *in vitro* totipotent cells have only been generated by a few groups,[2,3] pluripotent cell lines, with the capacity to differentiate into all the embryonic lineages, have extensively characterized. These cell lines can be generated by the ex vivo expansion of the blastocyst to make ESCs or through reprogramming to generate induced pluripotent cells (iPSCs). Pluripotent stem cell lines can also be derived from later stages of epiblast development, Epiblast stem cells, these cells exist closer to differentiation and are therefore said to be to primed.[4]

Cells that specialize further and hence become more limited in the number of specific cell types, they can give rise to, are called multipotent. Multipotency is a feature of adult stem cells, that sustain themselves through successive self-renewing cell divisions and are found within various types of tissue. Adult stem cells are

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thought to act as a resource for repair and homeostasis. In addition, by limiting the production of new cells to discrete population, the organism reduces potential targets for mutation in cancer. Their role is thought to be to maintain and repair the tissues they are found in and these cells have potential for use in tissue engineering via lineage-specific differentiation. The canonical adult stem cell is the hematopoietic stem cells (HSC), which give rise to all blood cells. The cells are defined based on their capacity to regenerate an entire hematopoietic system in response to injury. Neural stem cells can be isolated from both the adult and embryonic brain, and are able to give rise to neurons, astrocytes, and oligodendrocytes. Within the skeletal muscles, satellite cells act as progenitors that can produce new myoblasts that support normal growth and can aid in regeneration following injury. Stem cell populations have also been characterized in the skin, intestine and the germ line. There are also progenitor cells that give rise to bone, muscle and cartilage that are derived from mesenchyme. These so called Mesenchymal stem cells (MSC) probably represent different classes of progenitor, but are characterized by similar morphology and marker expression.

Considerable effort has been invested in understanding the molecular biology of stem cells. However, the maintenance of stable potent cell types through successive cell divisions is dependent on the niche with in which these unique cells reside. Interactions with this niche and with the cells and extra-cellular matrix that it is composed of are vital determinants of potency and self-renewal. These external, biochemical or mechanical interactions can trigger cellular responses, which may in return affect cell structure, stiffness, motility, function or gene expression.[5,6] Recent studies have shown that substrate stiffness,[7,8] cell shape[9] and mechanical stresses[10,11] are indeed linked to the commitment of stem cells to different lineages. As will be apparent from the current proceeding, the mechanical properties of stem cells have also been linked to the capacity of these cells to renew or differentiate toward specific lineages. The results from such studies indicate the importance of mechanical, as well as biochemical or biomolecular cues, indicating the importance of interdisciplinary research within the field of stem cell biology. Light is an excellent tool to investigate the real time mechanical properties of stem cells. The use of fluorescent reporter proteins has enabled imaging of the lineage choices made by stem cells. The focus of this proceeding is on the use of light to investigate the mechanical properties of stem cells, and how these properties change upon differentiation. The optical tweezers and the optical stretcher are two excellent tools for such investigations as they can operate on living cells suspended in an appropriate medium or matrix. When the appropriate laser wavelengths and laser powers are used, they are also nearly non-invasive.

2. INVESTIGATING CELLS BY OPTICAL TWEEZERS

Optical tweezers (OT) using a single tightly focused laser beam were first realized by Ashkin *et al.* in 1986, where they demonstrated stable trapping of microscopic particles.[13] Soon after, they demonstrated that microorganisms could also be optically trapped and would even stay alive and divide within the laser trap.[14] These results paved the way for the large success of optical trapping within the field of biophysics, where optical traps have, e.g., been widely used for mechanistic based studies of single molecules.[15,16] Optical tweezers are nearly non-invasive for biological specimen if the laser emits in the near-infra red regime and the laser power is kept low.[17,18] Optical twezers have been widely used for *in vitro* mechanistic single molecule studies, for instance of stepping size and forces exerted by molecular motors[19] and DNA force-extension properties.[20] Also, OT have been widely used to study living cells, for instance cell movement,[21,22] cell membrane properties,[23,24] and to characterize the viscoelastic properties of the cell cytoplasm.[25–27] OT can even perform accurate force measurements of single molecules inside living cells.[16,28,29] The simplest realization of an optical trap is just to have a laser beam tightly focused by, e.g., a high NA objective of a microscope. Figure Fig. 1**A** shows how an optical trap (orange line) can be incorporated into a confocal microscope (yellow lines refer to the confocal light),[12] and this combined platform is useful for simultaneous optical manipulation and visualization of fluorescently marked biological specimen.

The cell membrane serves as a barrier between a cell and its environment. Therefore, the membrane plays an important role for many cell functions and much communication takes place across this membrane. The membrane tension is considered crucial, e.g., for endocytosis, adhesion, motility, traffic across the membrane, and for attachment between the membrane and the cytoskeleton.[30,31] Hence, it is not a surprise that membrane function has also been linked to stem cell fate.[9] Membrane protrusions are normal for many cell functions, for instance during cell migration or while a cell investigates its environment.[24] Such membrane protrusions, can be 'artificially' extracted from a cell's membrane by attaching an optically trapped bead to a cell of interest (either

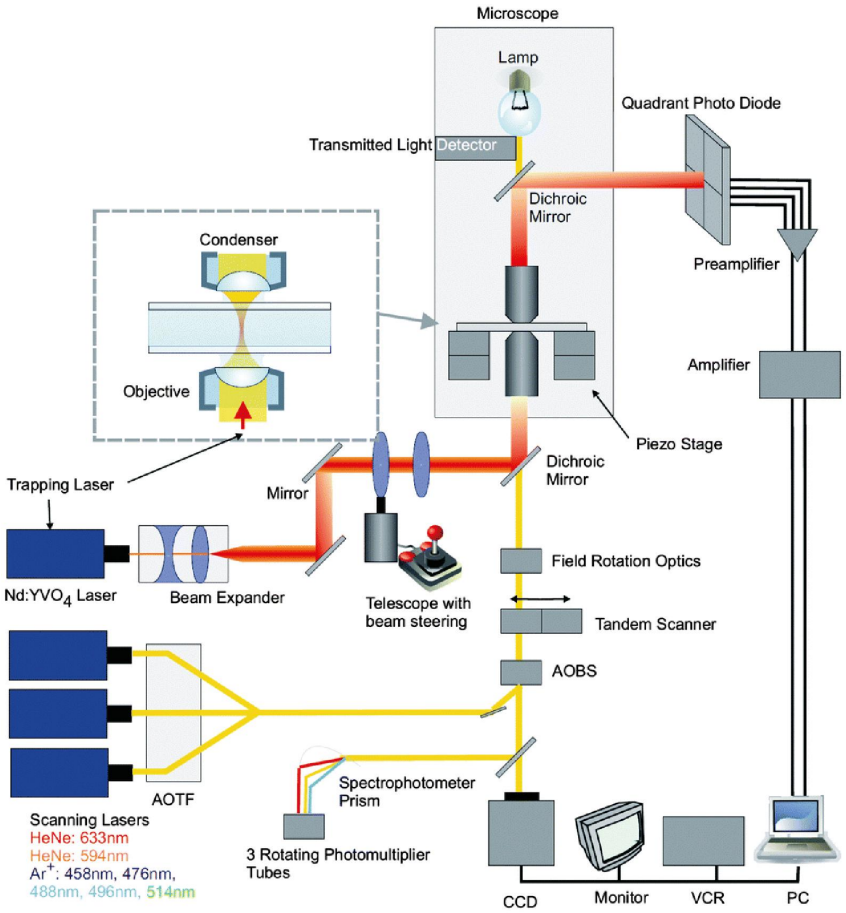

Figure 1. Sketch of a combined optical tweezers and confocal microscope platform. A near infra-red trapping laser beam (1064 nm, orange line) is expanded and reflected via mirrors into a telescope for beam stirring and for adjusting the laser focus in the axial direction. A dichroic mirror, which also transmits the confocal scanning laser light (yellow lines), reflects the trapping laser into a high NA objective, which focusses the trapping laser into a diffraction-limited spot and allows for simultaneous trapping and fluorescent confocal imaging. After passing the sample, the forward scattered NIR trapping laser light is collected by a condenser and another dichroic mirror reflects this forward scattered light onto a quadrant photo diode (QPD) for accurate positional detection with high temporal and spatial resolution. The inset shows a zoom-in on the sample region. The figure was reproduced from Richardson et al (2006)[12] with permission.

in a specific or unspecific manner) and pulling the bead away from the cell by the optical trap, as illustrated in Fig.2. This was done by Titushkin *et al.*[32,33] who extracted tethers by moving an OT at a constant rate of 0.7 μm/s away from human MSCs (see Fig. 2**A**). Simultaneously, they measured the force acting on the bead; the force abruptly increased upon releasing a tether from the cell's body (see Fig. 2**B**), upon further elongation the force fluctuated around a constant value, giving rise to well-defined force-plateau, and when the tether was approaching a certain maximum length, then the force abruptly increased until the bead escaped the trap (the tether did not break). This behavior indicates that the tether is formed from a membrane reservoir until it is depleted, which causes the bead to escape the OT upon further elongation. The average plateau force value was significantly higher and tether lengths were threefold longer for tethers pulled from hMSCs compared to tethers pulled from fibroblasts, indicating a difference in the membrane reservoir or maybe a difference in the connection between cytoskeletal elements and membranes[24] between the two cell types. Disruption of the cytoskeleton with cytochalasin D increased fibroblast tether lengths, while it did not affect hMSCs tether lengths. This indicates a different role of the cytoskeleton with respect to the membrane in the two cell types. The same researches investigated differences between hMSCs and osteoblasts using AFM and tether pulling experiments.[33] They found a distinct difference in cell elasticity and membrane mechanics which is likely to result primarily from

differential actin cytoskeleton organization in hMSCs and osteoblasts. Upon actin depolymerization or ATP deletion the authors found a significant increase on osteoblast tether length, but there was not effect on the hMSC tether lengths. These optical tweezers based observations lead the authors to suggest that actin plays a pivotal role in deteriming the mechanical properties of hMSC at the early stage of osteodifferentiation.

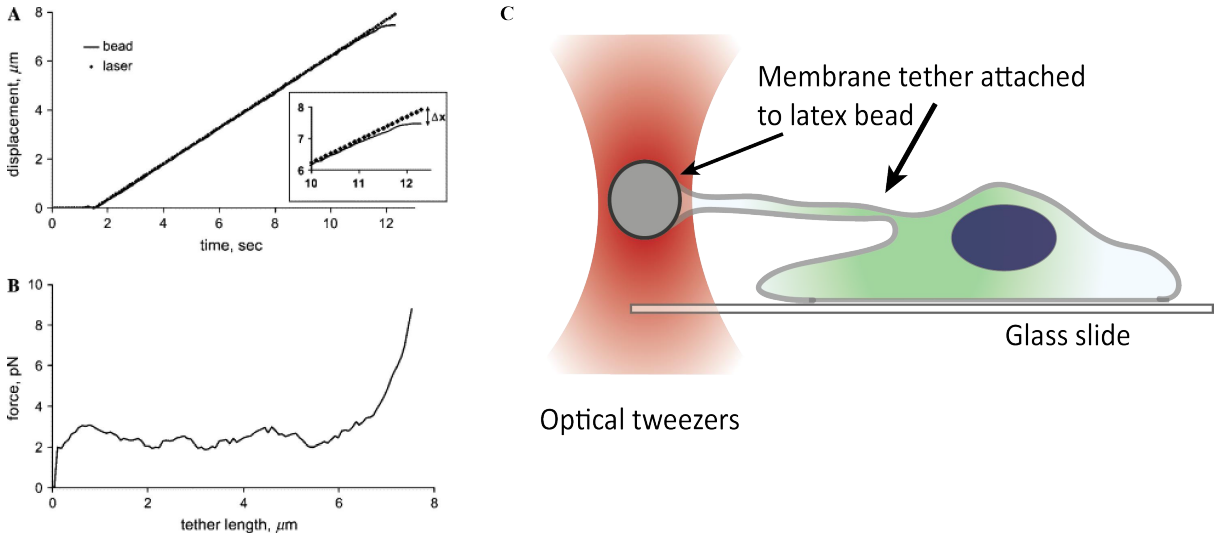

Figure 2. A stem cell tether extraction experiment performed by Titushkin and Cho.[32,33] **A** Displacement as a function of time of an optical trap (dots) being moved away from a cell and of a bead in the optical trap attached to a tether (full line). **B** Force on the bead as a function of tether length, between 0 and 7 μm the force fluctuates around 3 pN during tether elongation, this 'plateau force' is different for stem cells in different stages of their differentiation. **C** A sketch of the experiment. **A** and **B** are reproduced from *Biophysical Journal*, 90(7), Titushkin, I. and Cho, M., Distinct Membrane Mechanical Properties of Human Mesenchymal Stem Cells Determined Using Laser Optical Tweezers, 2582-2591, 2006, with permission from Elsevier.

Optical tweezers have also been successfully used to map out the rheology and viscoelastic landscape inside the cytoplasm of living cells. The rheological properties can be found, e.g., by monitoring the passive thermal motion of a tracer inside the matrix of interest.[34–36] Nearly all cells have endogenously occurring highly dense lipid granules with diameters around 200-400 nm spread throughout their cytoplasms, these granules serve as perfect passive tracers for studying micro-rheology of living cells. Using these endogenous lipid granules is less invasive for the cell than, e.g., using injected or endocytosed particles. Also, if using endocytosed particles, only the endocytotic pathway can be probed. Optical tweezers equipped with photodiode detection schemes (e.g., as shown in Fig. 2) can be used to trace the position of a single lipid granulus inside a living cell as a function of time, for this, the laser power need not be high enough to actually trap the granules, the light only serves to determine its position (see sketch in Fig. 3**A**). For longer periods of time it is advisable to use video tracking instead of quadrant photodiode detection albeit the resolution will be worse. From such a position timeseries, one can calculate the mean squared displacement (MSD) as function of lag time, Δ. The MSD is given as

$$MSD(\Delta) = \langle [\vec{r}(t+\Delta) - \vec{r}(t)]^2 \rangle \propto \Delta^{\alpha}. \quad (1)$$

The MSD characterizes the viscoelastic properties at the location of the lipid inside the living cell, the different categories are visualized in Fig.3**B** and described below:

$\alpha > 1$ The granule performs super-diffusion, an indication of active intracellular movement which could, e.g., be caused by molecular motor mediated transport or by the granulus being pushed by a polymerizing biopolymer.

$\alpha = 1$ A sign that the granule is undergoing normal Brownian motion, the motion characteristic of a purely viscous environment.

$\alpha < 1$ The granule moves in a sub-diffusive manner, the motion is likely to be constricted to to the cytoplasmic crowding. The closer α is to 1, the more viscous the environment, and the smaller α is, the more elastic the environment. Particles embedded in a polymer network exhibit sub-diffusive behavior.[37]

This 'passive tracer' OT methodology has been used to characterize the viscoelastic properties of *S. pombe* cytoplasm[25,38] and of human umbilical vein endothelial cells;[27] for both these cell types, the motion of the granules was found to be subdiffusive at short time scales (see Fig. 3**C**) and to display a larger variety of different types of motion at longer timescales. In addition, the interesting phenomena of weak ergodicity breaking was observed in time-averaged MSD curves for both these cell types.[27,39]

Optical tweezers exert a harmonic potential on the trapped particle, and if the spring constant can be accurately determined, the absolute force can be found, also inside a living cell. It is, however, not trivial to find the spring constant characterizing the optical trap inside a living cell or an organism, because the normal calibration methods rely on the passive thermal fluctuations of a trapped particle within a purely viscous environment, which is not exactly the case inside a living cell. As a result, the trap must be calibrated in the exact same environment as the force measurements, otherwise it is not reliable. To overcome this challenge, and to perform a reliable force calibration inside the visco-elastic complex landscape inside a living cell, a combination of passive and active measurements can be used, where for the active part, the bead or the sample is oscillated with respect to the laser in a controlled fashion while monitoring the relaxation of the trapped object into its equilibrium position, thereby obtaining the retardation spectrum.[40,41] This method is based on the fluctuation dissipation theorem and also allows for extraction of both the trap stiffness and the shear storage (G') and the shear loss (G") moduli, which characterize the viscoelastic properties of the cell. For similar investigations of stem cells, optical trapping combined with fluorescent imaging offers the potential to couple physical measurements with the simultaneous measurement of expression of fluorescent reporters.

3. INVESTIGATING CELLS BY OPTICAL STRETCHING

Another successful technique to investigate viscoelastic properties of cells is the optical stretcher (OS).[42] The primary principle behind trapping optical stretching is, just like optical trapping, momentum transfer. To create an optical stretcher, one uses two counter-propagating aligned non-focused laser beams (see Fig. 4**A**). The total force acting on a dielectric object placed between the opposed laser beams is zero, however, if the object is elastic enough, it will experience stretching along the laser beams' axes due to the momentum changes internally inside the object. An OS does not require a handle, it simply stretches the cell itself. OS has been used to investigate the elasticity and compliance of various cells, for instance fibroblasts and red blood cells.[42] More recently, optical stretching in combination with AFM measurements were used to investigate the mechanical properties of stem cells.[43] They traced the mechanical changes on the surface of attached MSCs during extended passaging and found that both the cytoskeletal organization and mechanical stiffness of the attached MSCS populations were strongly modulated over more than 15 population doublings *in vitro*. These mechanical changes were not observed with suspended MSCs, thus indicating the cells attachment to a surface, or lack thereof, is a decisive determinant of its cytoskeletal structures.

Differentiating blood cells have also been investigated using an optical stretcher;[44] it was demonstrated (see Fig. 4**C**) that the stiffness of HL60 human myeloid precursor cells is significantly different from the stiffness of their differentiated lineages: neutrophils, monocytes and macrophages. These three lymphocytes also show significantly different compliances among themselves: On short time scales, neutrophils were the most compliant, followed by monocytes, the undifferentiated precursor cells, and macrophages. At longer time scales however, macrophages become more compliant. These observations comply well with their biological functions; neutrophils and monocytes have to deform fast to squeeze through blood capillaries with high blood flow to get to their target, while macrophages migrate through tissues over long time scales.

Using embryonic stem cells, an optical stretcher was used to investigate how the mechanical properties of the cells correlate with the expression of a transcription factor associated with pluripotency, Nanog.[45,46] Mouse ESCs containing green fluorescent protein reporter under the control of the Nanog promoter were used to distinguish what the authors refer to as a high Nanog population, that are thought to represent stable undifferentiated ESCs, and a Nanog low population that was believed to correspond to cells that, while still ESCs, had just begun to

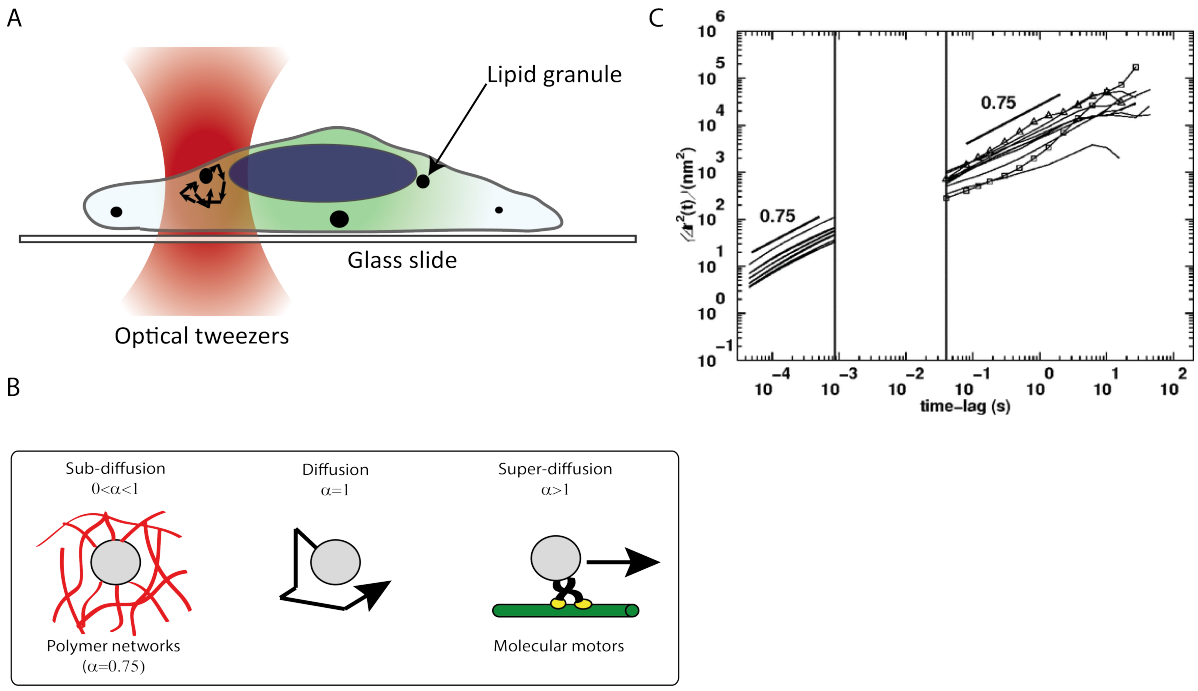

Figure 3. Mapping out the viscoelastic landscape within living cells. **A** Sketch of how optical tweezers are focused onto an endogenously occurring lipid granule within a cell and trace the granules thermal fluctuations in order to determine the viscoelastic properties at the location of the granulus. **B** Illustration of how α can be used to characterize the diffusive behavior of a tracer. **C** Mean squared displacements of lipid granules inside living *S. pombe* yeast. At short time scales, the tracking was done using optical tweezers in combination with photodiode detection, on longer timescales, the detection was video based. On short timescales the granule motion was always subdiffusive, however, on longer timescales a larger variety of diffusional modes was observed. **C** is reproduced with permission from I. M. Tolić -Nørrelykke, E. L. Munteanu, G. Thon, L. Oddershede, K. Berg-Sørensen, Physical Review Letters, 93(7), 078102, http://link.aps.org/doi/10.1103/PhysRevLett.93.078102 , 2014. Copyright (2016) by the American Physical Society.

differentiate. These cells are referred to as primed, as they had begun to differentiate, but could still return to the stable ESC state if left in culture for a time. They found that the low Nanog population was significantly more compliant than the high Nanog population, and the low Nanog population retained this higher compliance even after disruption of the cytoskeleton, suggesting that these changes were not due to cytoskeletal differences. They also found that cells in the Nanog high state had a significantly stiffer nucleus that they associated with a higher degree of chromatin condensation. The results suggest that chromatin condensation could be responsible for the mechanical differences seen between the high and low Nanog high populations[45] and could also be a factor involved in the initiation of differentiation.

4. CONCLUSION AND OUTLOOK

In the past 15 years, many studies have shown that mechanical cues, both external like shear stress and substrate stiffness and internal such as the cell's cytoskeleton, influence the commitment of stem cells to certain lineages. It is also becoming more and more evident that stem cells change their mechanical properties during differentiation. To understand these mechanisms, more effort will need to be put into mechanistic based biophysical investigations of stem cell properties. For these types of investigations, optical based methods, such as optical tweezers and optical stretchers, are an essential tool as they can be used on and within living cells and are nearly non-invasive. As a result, we anticipate an extremely important role for these techniques in deconstructing comprehensive mechanisms regulating the choices that stem cells make in both differentiation and self-renewal.

5. ACKNOWLEDGEMENT

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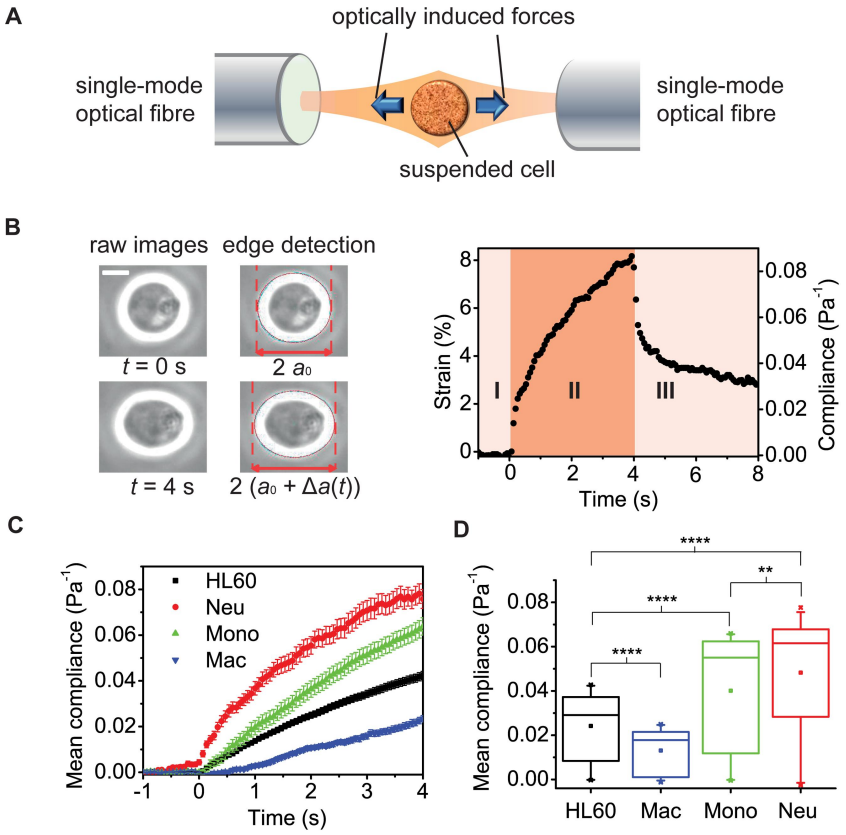

Figure 4. Optical stretching of differentiating blood cells.[44] **A** Sketch of optical stretcher based on two counter-propagating laser beams. **B** Images of a cell trapped in an OS at low optical stress at t= 0 s and stretched due to increased optical stress at t= 4 s. The scale bar in the first image is 10 μm. The right graph shows the strain and compliance profile of the pictured cell (I: before imposing optical stress, II: during the stress period, III: after relieveing the stress). **C** The compliance profiles of the undifferentiated precursor cells, HL60, and the three differentiated lineages: neutrophils, monocytes and macrophages. **D** Average compliance during the 4 s stretching interval. Statistical differences are indicated by asterisks, ** stands for a p value of <0.01 and **** for a p value of <0.0001. Figure was reproduced with permission from Ekpenyong et al (2012) Viscoelastic Properties of Differentiating Blood Cells Are Fate- and Function-Dependent. PloS ONE 7(9): e45237. doi: 10.1371/journal.pone.0045237 .

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