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Historical Perspective

Functional blood cell analysis by label-free biosensors and single-cell technologies

Zoltán Szittner *, Beatrix Péter, Sándor Kurunczi, Inna Székács, Robert Horvath

Nanobiosensorics Laboratory, Centre for Energy Research, Institute of Technical Physics and Materials Science, 1121 Budapest, Hungary

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ABSTRACT

In this review we aim to summarize the current state of methods for label-free identification and functional characterization of leukocytes with biosensors and novel single cell techniques. The growing interest in this field is fueled from multiple directions, with the different aspects highlighting benefits of these novel technologies in comparison to classical methods. The advantage of label-free characterization is that labeling the cells might affect their behavior, and therefore lead to a biased description of the investigated biological phenomena. Label-free biosensors can offer the benefit of (i) decreasing processing time and reagent costs, (ii) enable point-of-care diagnostics, and (iii) allow downstream application of the investigated cells. Moreover, (iv) label-free detection allows the monitoring of real-time kinetic processes, opening up new avenues in contrast to traditional structural characterizations.

The emphasis in the review will be on techniques on the characterizations of single cells with special attention to surface sensitive technologies. Recent developments highlighted the importance of small cell populations and individual cells both in health and disease. Nonetheless techniques capable of analyzing single cells offer a promising tool for therapeutic approaches where characterization of individual cells is necessary to estimate their clinical therapeutic potential. Most of the approaches discussed here will cover the cellular activation, adhesion as measured on functionalized solid substrates, since this approach offers the most advantages. Analyzing various cells on solid substrates not only allows their individual morphological characterization and therefore a more precise description of their activation, but as well offers an opportunity to design multiplex measurements. With this approach different stimuli can be investigated in parallel and measure cellular avidity to targets, an important aspect of gaining more and more attention recently in characterization of T-cells and antibody effector functions.

Finally, novel label-free approaches provide a solution to extracting unlabeled cells for downstream processing (e.g., transcriptome analysis, cloning or the aforementioned clinical potential), where ongoing and potential further applications are discussed.

1. Introduction

Immune monitoring assays provide insight into the state of an individual's immune system [1]. This information is essential to determine the efficacy of various immune therapies to treat malignancies, autoimmune diseases and cancer, to understand changes of the immune system during infectious diseases, as well as to monitor the effect of vaccination. There is a clinical need for standardized test for this purpose [2]. Deciphering immunological parameters is necessary to reach personalized medicine [3]. Peripheral blood is the most commonly used sample source, where cells, proteins, metabolites, etc. are readily available for testing. In order to minimize assay variation, in an ideal setting, it is suggested to apply minimal sample preparation and also reducing the variety of reagents, labels as much as possible. Label-free biosensors with the capability to monitor single cells seem perfectly fit for such measurements and offer the chance to characterize immunological reactions solely based on the intrinsic properties of live cells in during their interaction with various stimuli on the sensor surface. In this review we provide an overview on recent advances in this field.

The importance of cellular heterogeneity found in blood is well established in hematology and oncology ever since the first blood film based diagnostic approaches emerged [4]. Laboratory analysis of blood

* Corresponding author.
E-mail address: zoltan.szittner@gmail.com (Z. Szittner).

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composition is a centerpiece of clinical routine tests and is mainly performed by automated cell counters and hemoanalyzers [5], based on the light scattering properties of single cells. Leukocyte recognition and typing is on the verge of full automation, based on image analysis of the classical blood smears [6–13]. Recent advances, however, provide further advancement in the label-free preparation and analysis of blood-smears [14]. Label-free hematological analysis has provided multiple approaches for further automation of this process, however, solely based on autofluorescence these methods not ready to provide the precision for clinical diagnostics [15].

When further analysis is necessary flow cytometry based immunophenotyping [16] is the main approach next to the classical blood smear based morphological analysis. While the classical methods reveal the heterogeneity in white blood cell morphology and identify various abnormalities, they lack the capability to study the function of the cells. Flow cytometry (hereafter FC), however, offers multiple different functional assays [17]. The main limitation of FC is the necessary use of labels, which are also an important limiting factor for investigations outside of well-equipped laboratories, where recent advances currently allow over 30 parallel fluorescent channels in FC to be used. This number can be possibly further increased, however, the reagent costs and trained personnel requirements may limit its use outside of specialized laboratories. Most FC assays are performed in solution, while activation of white blood cells mainly occurs on the surface of substrates, where adhesion of the cells plays a crucial role in their activation [18].

In an ideal study setting, immune monitoring is based on the measurement of all its components' interactions with various ligands or their combination on a positional microarray. Here, all constituents of blood could possibly react with each ligand (for example peptide-MHC ((Major Histocompatibility Complex) complex as a ligand for T-cells, or antibodies binding to substrate attached antigens as a ligand for various antibody dependent effector cells). Since the theoretical number of ligands and their combination is limitless, the positional array format seems necessary for assays where a large number of ligands are to be studied. Optimally such an approach would also need to be label-free in order to minimize the chance of interfering with the ligands' functionality.

Primarily in immunology, antibodies are used to label cell surface markers, however, their tendency to bind to certain cell populations is well known and therefore it is important to note that these might interfere with immunophenotyping, even when compared to matched isotype controls in flow cytomettry [19] and immunohistochemistry as well [20].However, single-cell-based approaches are already available for immunology as well [21]. Through micromanipulation, single cells can be isolated in microfluidic systems, for example, where various biosensors can be used to detect cells, their activation, or secreted proteins [22–24]. Label-free biosensor approaches provide further advantages since the activation of the cells can be measured in a kinetic manner; moreover, these methods also have the potential to study cells in a nondestructive way and thus enable downstream analysis and further processing of the cells with other techniques of interest. On the molecular level, recent developments in mass spectrometry enable label-free proteomic and metabolomic studies as well, even from a single cells [25,26].

With the arrival of the transcriptomic era the importance of single cell analysis gained further momentum. Single-cell transcriptomics revealed further complexity of leukocyte subpopulations and now one of the main challenges is to reduce this complexity through biomarkers to explain certain clinical conditions, highlighting the need for biosensor based approaches to keep up the pace in order to provide further insight by phenotypical characterization of the investigated cells.

Microfluidics provides an excellent tool for separating subpopulations of white blood cells, isolate them, and study them on a single-cell level. Recent developments in these immunoassays mainly focused on miniaturization and point-of-care realization of well-established techniques. Most microfluidic assays still utilize labels, such as antibodies or fluorescent reporter molecules, and therefore increase complexity and preparation time of the assay and possibly interfering with the observed phenomena. Microfluidics has greatly contributed to the single-cell studies, including droplet microfluidics in scRNAseq and isolation rare cell populations, such as circulating tumor cells from liquid biopsies [27]. Moreover, separation techniques based on cell size, density and stiffness make microfluidics an important component of point-of-care (POC) lab-on-a-chip applications. In many cases, the biosensors are already integrated into microfluidic platforms [28] or into novel solutions where flow conditions over the adhering cells can be easily tuned in a high-throughput manner [29]. For example, using microfluidics, Gopalakrishnan et al. fabricated a microsystem that allowed them to monitor the migration and chemotaxis of immune cells in real time [30], and microfluidic approaches provide further technological advances to study immune cells [31].

The development of biosensors, transducers, and assay surfaces is entering its stage where single-cell studies are possible even in a label-free manner, as supported by the various techniques and pre-designed assay surfaces [32–34]. We think that at this stage the next important step in immunological applications by using these novel live cell assays is to gather information on the clinical manifestation of various patient groups, find strong correlations when comparing measured parameters of immune cell activation with established gold standard methods, and thus enable the decentralization of functional cell based diagnostics. Cells-based biosensors have recently gained the center of attention and offer the benefit of studying single cells, their response to various signals and compounds, their secretory activity. Recent reviews discuss key results in the field of cell-based sensors [35,36].

Biomarkers are of emerging importance. While by their nature genetic, biochemical, cellular, histopathological, clinical or imaging-based biomarkers, based on their utility, can be diagnostic, prognostic, predictive, and pharmacodynamic [37]. It is important to point out that the majority of the currently applied biomarkers are of molecular nature; however, cellular phenotypic biomarkers are based on intrinsic biophysical properties of the cell and are therefore suitable for label-free immune monitoring.

It is important to make a distinction between the two main directions of biosensor studies. Miniaturization and simplification of various immunological assays paves the way for POC applications, and thus, by increasing availability, these techniques have the potential to expand our current understanding of various biomarkers in larger sample groups, and further enlighten the heterogeneity in immune status within individuals and populations. These approaches focus on the detection of antigen specific antibodies and cytokines, as discussed in detail below. By potentially expanding the scope of these miniaturization efforts, these techniques offer great benefit by increasing the availability of measurements already performed in the laboratories. However, these techniques lack the potential of further characterizing immune status beyond the limitations of current techniques. On the other hand, biosensors have the potential to help us identifying novel biomarkers of cellular phenotypic nature. This is especially important in the multi-omics era of immunology; however, biosensors are not yet considered as a mainstream application in this field and are mostly confined to research laboratories [38]. Another direction in the field is the immune organ-on-a-chip approach, however, due to complexity of generating the cellular architecture, these approaches, at least for now, are extremely difficult to adopt outside of laboratory [39]. We also believe that phenotypical characterization of single cells, their responsivity and activation on different substrates could be established in low-resource settings in the form of POC setups [40]. Similarly to recent developments in the selection of medical treatment as seen in pharmacogenetics, decision support tools could expand their scope and offer additional support and enable evidence-based medicine in further areas [41].

In this review we overview the most important optical, electrical and

mechanical label-free technologies having the greatest potentials in immune cell studies and application developments. We give special attention to surface sensitive methods, as well as the functionalization of transducer surfaces for a specific biological or biotechnological application. Novel solutions capable of selecting and isolating individual cells from a larger cell population are also discussed, by mainly focusing on techniques which are minimally invasive, thus allowing further processing of the targeted cells. We also review recent data from label-free solutions to characterize and/or identify leukocyte subpopulations and their activation response to stimuli by various ligands.

2. Technological overview of label-free biosensors

Traditionally biosensors consist of three main subunits: (i) the biologically active ligands (receptor, enzyme, aptamer, etc.), that enable binding of the analytes, such as proteins, metabolites, DNA, bacteria or even whole mammalian cells in a specific manner. (ii) The detector or transducer element that transforms the biological response into a measurable output signal, and (iii) a suitable signal processing unit that produces the final biosensor data [42]. Biosensors can be also categorized based on the analyte, biological ligand, type of transducer, and field of application. Briefly, based on the type of transducer, electrical, optical and mechanical biosensors were developed with each type consisting multiple slightly different approaches [32,42–44]. Schematic illustrations of label-free optical biosensor technologies are shown in Fig. 1, electrical methods are in Fig. 2 and the mechanical ones are in Fig. 3.

Biosensor spatial resolution is an important parameter because it determines whether population, single cell, or subcellular characteristics and responses can be studied. Although microscopy techniques are classically not considered to be biosensors, these approaches allow us to decipher the cellular heterogeneity and identify cells also in biosensor-based measurements. Flexibility and availability make these techniques highly important and can be easily combined with biosensors and used to confirm the results [45]. When considering blood as a complex mixture of analytes or purified white blood cells (WBCs) identification is a great challenge. Multiple different strategies based on label-free imaging techniques have recently showed that these methods are essential and capable of correctly identifying leukocyte subsets. Most importantly, molecular imaging with deep-ultraviolet microscopy was presented as readily available for complete hematology analysis, which could also pave the way for POC applications and a new era of hematology analyzers [14]. Other autofluorescence-based imaging methods, such as fluorescence lifetime imaging microscopy (FLIM) [46], have the advantage of subcellular resolution, allowing the monitoring of differentiation, redox state and metabolism of the investigated cell thus providing an excellent platform to characterize leukocytes [47]. Digital holographic microscopy through quantitative phase contrast imaging is another promising technique that has the ability to monitor multiple morphologic parameters and motility of the investigated cells [48] (see Fig. 1A). This technology is now available in a highly miniaturized format, suitable for in situ live cell measurements inside a humidified incubator [49–51]. Cellular parameters like motility, migration, proliferation rate, and morphological parameters such as optical thickness, volume and cell area can be measured in a straightforward manner, and these parameters for large cell populations can be investigated. The technology was already demonstrated in small molecular studies [49], label-free toxicology [51] and in cell invasion studies [50]. Most label-free optical biosensors are based on refractive index and allow the kinetic measurement of molecular and cellular interactions [52]. These biosensors are considered among the most successful biosensors for studying molecular interactions, and surface plasmon resonance (SPR) and waveguide-based biosensors (Optical Waveguide Lightmode Spectroscopy (OWLS), Resonant Waveguide Grating (RWG)) are applied to

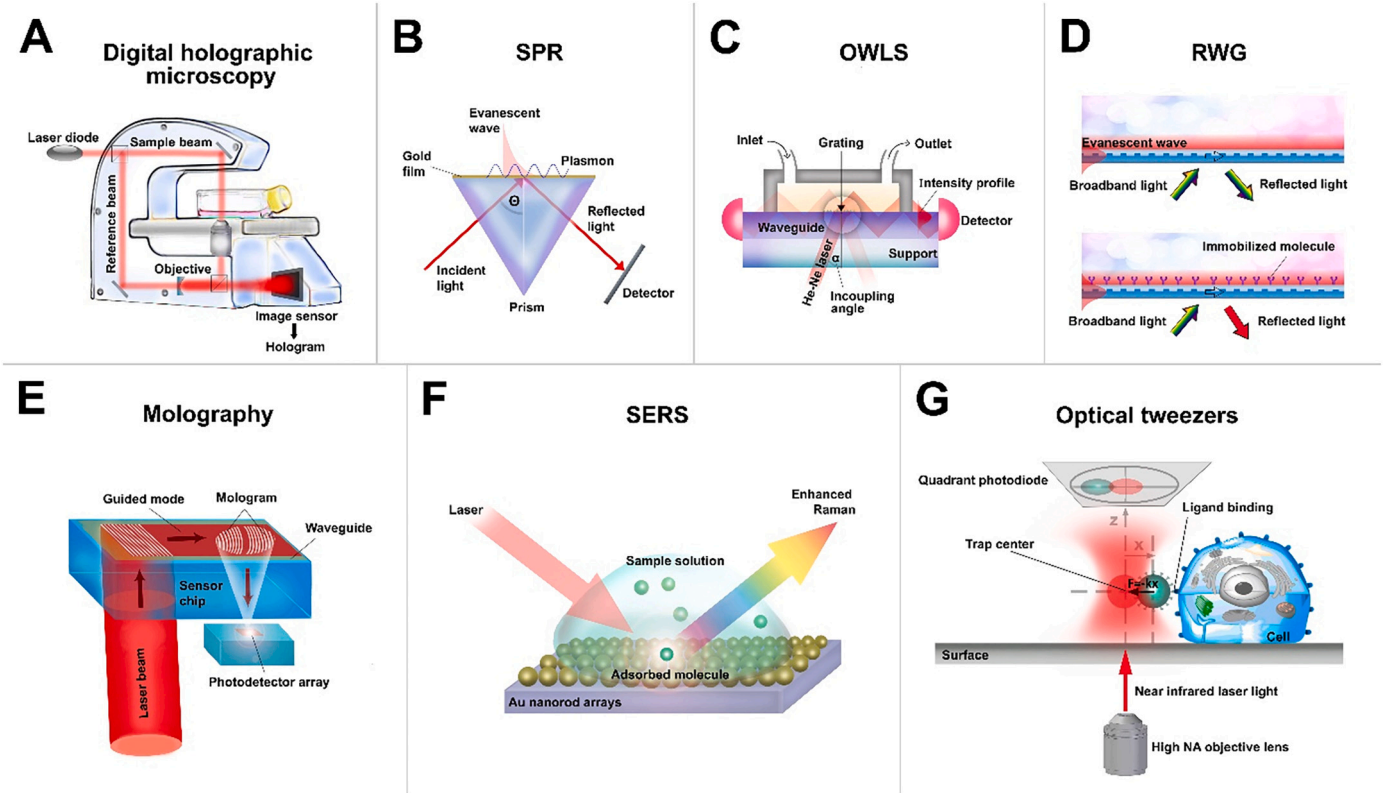

Fig. 1. Label-free optical biosensor technologies for cellular studies. Schemes show working principle of A. digital holographic microscopy, B. surface plasmon resonance (SPR), C. optical waveguide lightmode spectroscopy (OWLS), D. resonant wavelength grating (RWG), E. mology, F, surface enhanced Raman spectroscopy (SERS) and G. optical tweezers. Figures adapted from [32,44].

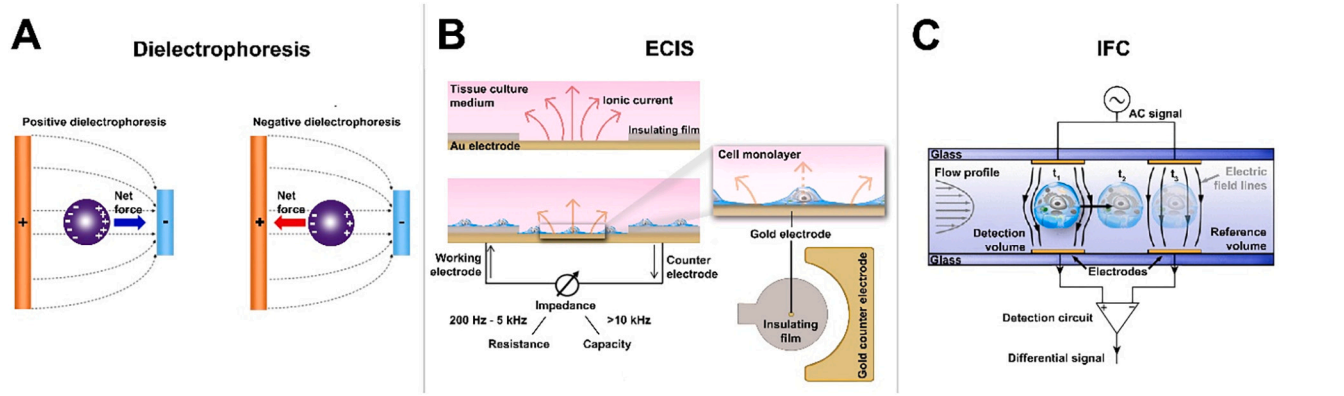

Fig. 2. Label-free electrical methods for cell analysis. Schemes show working principle of A, dielectrophoresis, B electric cell-substrate impedance sensing (ECIS) and C. impedance flow cytometry (IFC). Figures adapted from [85–87].

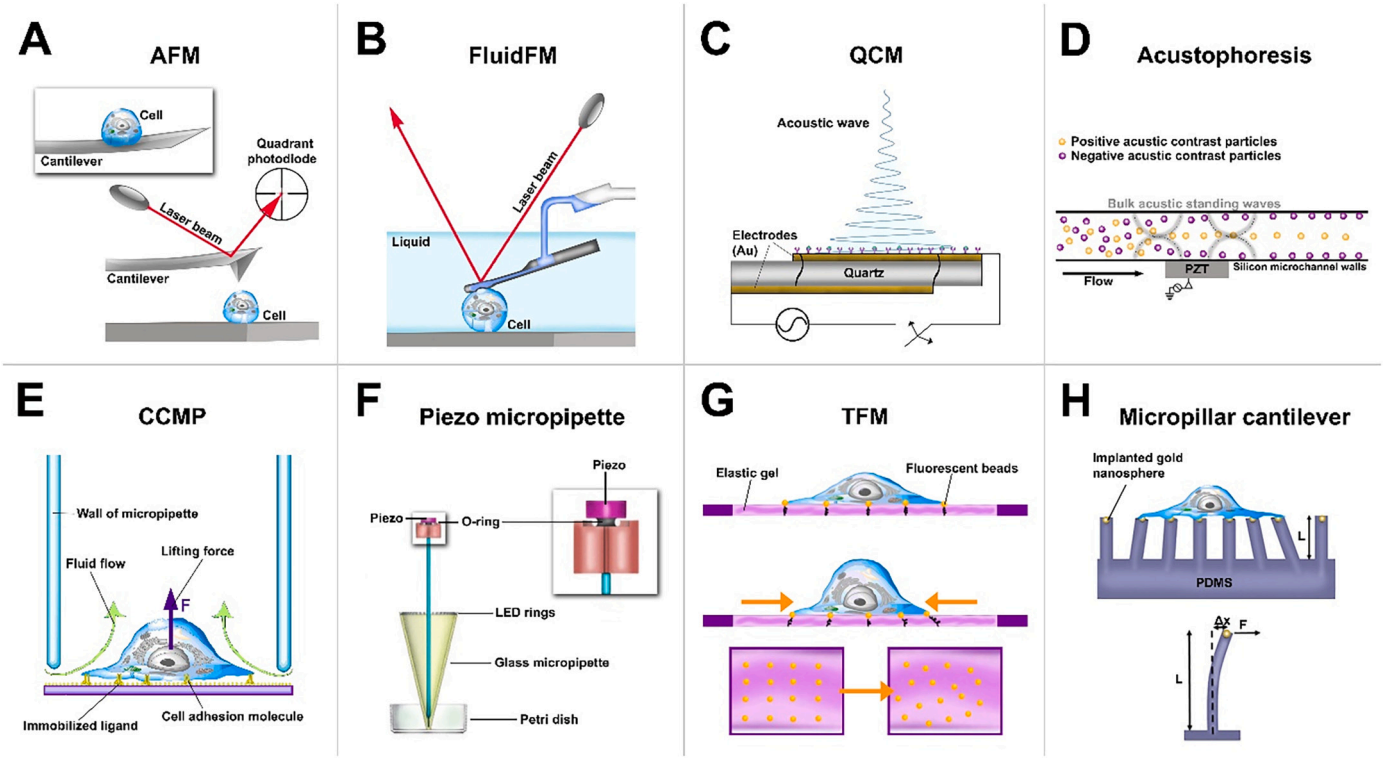

Fig. 3. Label-free mechanical technologies for cellular studies and single-cell targeting and isolation. Schemes show working principle of A. atomic force microscopy (AFM), B. fluidic force microscopy (FluidFM), C. quartz crystal microbalance (QCM), D. acustophoresis, E. computer controlled micropipette (CCMP), F. piezo micropipette, G. traction force microscopy (TFM), and H. micropillar cantilever. Figures adapted from [32,44].

studying whole cells as well [32,53–58]. Among these SPR and RWG based optical sensors are in the most developed stage with multiple different approach being very close to clinical application, mainly in the detection of antigen specific antibodies [59]. An important new development is molography (see Fig. 1E), allowing molecular kinetic analysis even inside the cells. In molography, receptor molecules are arranged in a special nanopattern and using a suitable optical readout the binding signal from this pattern is read out, significantly reducing the signal of nonspecific binding events [60–63].

RWG technique employs an evanescent electromagnetic field to sense changes in the local refractive index within the sensing volume (~150 nm) close to the sensor surface [64]. The integrated response profiles of whole cells can be followed kinetically in a high-throughput format using biosensor microplates. This technique has already become widespread and has been applied for the discovery of cell-to-surface adhesion kinetics [65], cell adhesion and detachment at different flow velocities [29], finding out the role of cancer cell glycocalyx during the adhesion process [66], and quantification of integrin–ligand binding affinity in intact living cells [67,68]. It can also be easily applied to monitor cytotoxicity [51,69], tracking small molecule binding [70], and real-time kinetics and dynamics of nanoparticle interaction with cells [71]. Schröder et al. presented how this technique can be applied to monitor dynamic mass redistribution of adherent cells in response to various known agonists of the G protein–coupled receptor (GPCR) and thus allows dissecting complex GPCR signals [72].

In addition to the detection of adherent cell signaling of different cell lines [64,73] and primary endothelial cells [74], this technique was successfully applied to follow signaling events in non-adherent cells as well. Receptor-triggered integrated cellular responses were studied in different B cell lines [75,76], primary human neutrophils [77] and

human primary B cells [78].

This technique has also shown promise at the single-cell level detecting spheroidal cancer cell invasion [79]. Recently, the RWG biosensor was combined with robotic fluidic force microscopy (FluidFM, the instrument is also called FluidFM OMNIUM) for surface adhesion force calibration in single cells [33]. This development allowed for the measurement of cell adhesion force kinetics of large cell populations for the first time. Importantly, surface plasmon based technologies including Surface Enhanced Raman Spectroscopy (SERS) also have the potential of POC applications [80]. However, in most cases the clinical accreditation is still missing, therefore validation criteria is to be tightly followed when considering marketing such applications [81]. Optical tweezers are considered as one of the most sensitive methods for measuring forces in a liquid environment, by exploiting the optical forces on dielectric materials inside the focal spot of a laser beam. Force measurement in the pN range is perfectly suitable using this technique, even allowing the characterization of the forces of individual biomolecular events [82,83]. Using special microtools and multiple laser spots, novel arrangements were also demonstrated to measure cell adhesion forces and cell mechanical parameters [84].

Impedance-based technologies allow label-free characterization of adherent cells in electric cell-substrate impedance sensing (ECIS) and as well cells in solution in impedance flow cytometry (IFC), based on their electrical properties [88]. This versatile technique has been shown to be valuable in combination with microfluidics to separate certain subpopulations of leukocytes and enrich circulating tumor cells (CTC) [88]. Furthermore, cellular activation and differentiation can be monitored, also with cells in solution [89]. For adherent cells ECIS can be used to monitor viability [90,91], changes in adhesion [92], morphology, proliferation, migration, spreading of the cells [93,94], their response to pharmaceutical compounds [95] and to viral infection [96].

Similarly, dielectrophoresis (DEP), both insulator- and electrode-based, has been developed to separate cells based on their dielectric activity. Due to the lower field strength, insulator-based DEP offers a higher viability and, therefore, offers significantly longer analysis time while preserving integrity of the cells [97]. Importantly, DEP based technologies are also applicable on a single-cell level, offering another approach to prepare cell suspensions for single-cell analysis [98]. Zhang et al. demonstrated that DEP allows for a detailed characterization of single cells through determining biophysical parameters such as cell radius, specific membrane capacitance, and cytoplasm conductivity [99]. For further details on DEP we recommend the recent review of Henslee [100].

Biosensors based on mechanical principles allow sensitive monitoring of cellular and molecular interactions. Here the readout is based on mechanical deformation sensed through changes in electrical resistivity in the semiconductor or displacement of the light in the sensor [101]. After calibration, such techniques provide a straightforward answer to the cellular response to a ligand of interest. Multiple different platforms were developed to study the cell-substrate interaction on a single cell level [102]. Notably, traction force microscopy was the first technique that exploited elastic properties of various hydrogels, such as polyacrylamide gels, to monitor the forces exerted by cells on extracellular matrix components for example [103]. Here, fluorescent markers can be used to determine the forces exerted by cells on the substrate [104]. Micropillar based methods followed, where bending of the functionalized micropillars is readily measurable. Importantly this allows monitoring in a completely noninvasive manner [103].

Single cell (and molecule) adhesion force can be studied using functionalized microcantilevers, typically using atomic force microscopy (AFM) [105] in single-cell force spectoscopy [106]. However, the throughput of these methods is relatively low. Incorporation of a microfluidic channel can greatly improve the speed of data acquisition [33]. As avidity sums up the binding force in between receptors of the cells and their ligands, these techniques provide an important tool for studying these interactions. Further techniques to study single cell adhesion force are discussed in detail in the recent review by Ungai-Salánki et al. [44] Shinde et al. [107] and Saffioti et al. [18].

Piezoelectric crystal based sensors enable sensitive monitoring of ligand receptor interactions. Mass accumulation on the sensor surface results in a decrease in the frequency of the applied acoustic wave resonators. Quartz crystal microbalance (QCM) was one of the first biosensors developed on this principle [108,109]. This technique was extensively applied to determine cell-extracellular matrix interactions, typically adhesion through the integrins of the cells [110,111]. Recent developments allow monitoring of motility, proliferation, signaling, and morphology as well [112].

Recent results demonstrated an additional application of acoustic waves to characterize and separate cells. Urbansky et al. showed that acustophoresis allows multiplex separation of leukocytes into the three main subpopulations, in a high-throughput, label-free manner [113]. This approach was less efficient in regard of purity of the monocyte fraction, however, Hu et al. showed that combining optical with acoustic biosensors the purity of these fractions can be raised to over 95% [114]. By applying acoustic forces on single T cells, Kamsma et al. reported that T cell avidity and adhesion kinetics [115] can be determined by acoustic force spectroscopy [116]. (This principle is also applied by Lumicks to determine avidity of T cells and NK cells). Advances in acoustic micromanipulation was reviewed recently by Akkoyoun et al. and the technology shows promising results separating, sorting and patterning particles and cells as well [117].

The mechanical properties of the cells are of increasing interest. Next to morphology, biophysical properties of the cells, such as deformability, mass and stiffness, can as well be applied to characterize the activation state of certain leukocyte subpopulations [118–121]. In agreement with others [120], we believe that development of platforms allowing content-rich multiparametric biophysical cytometry in a nondestructive manner shows tremendous potential in biomarker discovery. In our view, these parameters are particularly important targets for investigation. The mechanical properties of the environment surrounding the cells are as well of great importance. Mechanotransduction, through sensing for example substrate stiffness, was shown to influence the activation state of the cells and therefore should be considered as an important experimental parameter when studying cellular activation [122]. This notion fuels the emerging field of mechanoimmunology as well [123].

Typical measurement results by employing novel label-free techniques (RWG, digital holographic microscopy, computer controlled micropipette and FliuidFM) are shown in Fig. 4.

3. Surface functionalization

These properties can be modified during sensor design and application of various substrates. Substrates may vary in the degree to which they resemble the in vivo environment. Also, their stiffness may be different, just as their ligand binding capacity, for example, in the case of hydrogels [126]. Song et al. in their recent paper discuss biosensor interface design and substrate options in detail [127]. However, for each sensing principle a different approach should be considered [126,128,129], ranging from biomimetic membranes to hydrogels.

Functionalization of the sensor with the ligands of interest is highly dependent on the nature of the ligand and the applied solid support [130]. Ranging from simple adsorption to covalently attached ligands and affinity-based oriented ligand deposition, many different strategies are available for ligand immobilization [131]. Examples for antibody immobilization onto the sensor surface are shown in Fig. 5. It is important to keep in mind that the different immobilization strategies may abolish the biological activity of the ligand. For details on this topic, we recommend the review by Asal et al. to the reader [132]. The advancements in the implementation of dynamic surfaces offer a highly useful tool by enabling the modification of the substrate and the ligands applied in the biosensor. These surfaces allow changing hydrophobicity

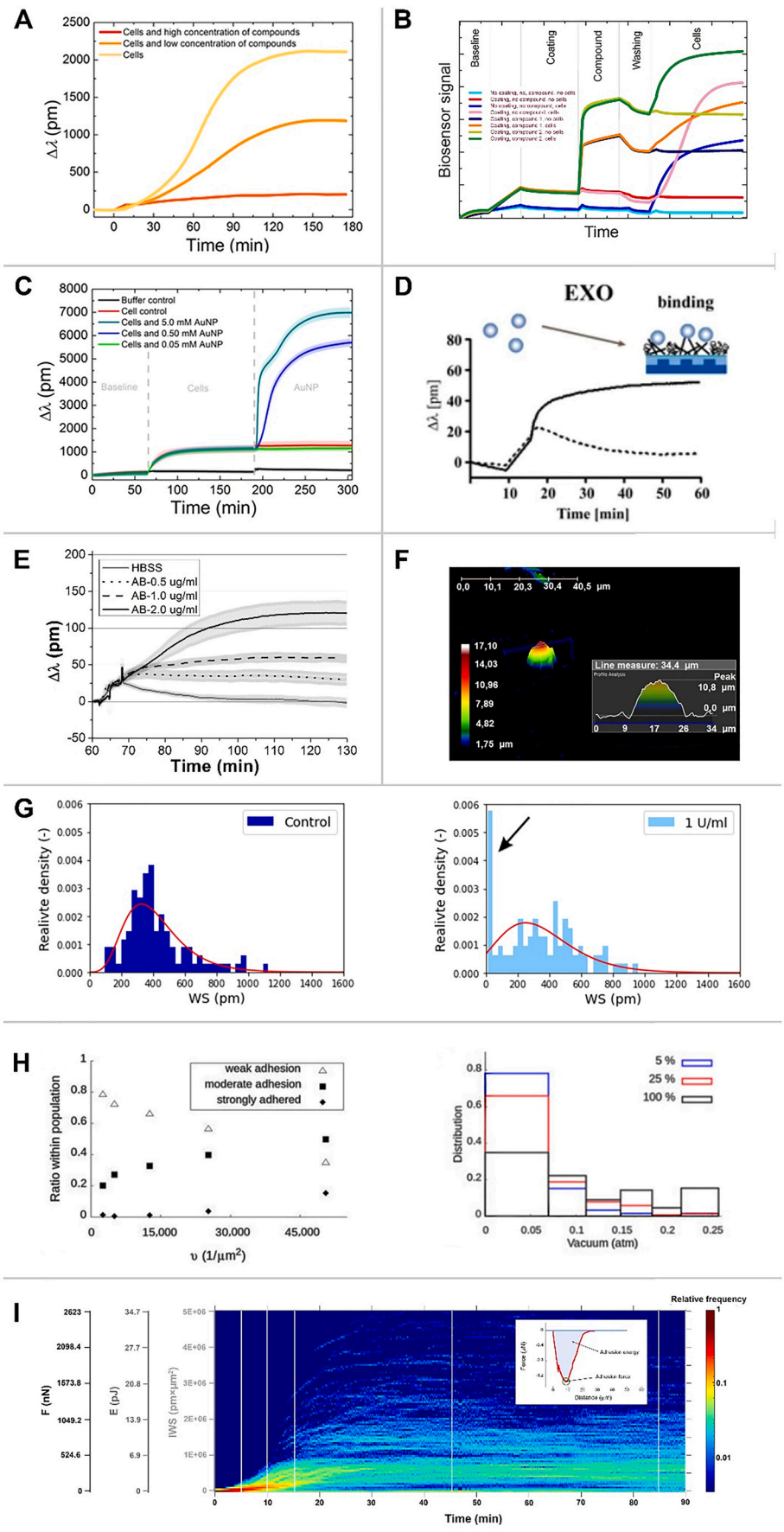

Fig. 4. Typical measurement results by employing novel label-free techniques. A, Real-time cell adhesion kinetic curves received by RWG biosensor. The effect of a certain compound on cell adhesion is measurable very easily; the kinetic curves show the inhibitory effect in a concentration dependent manner [67]. B, The adsorption of a compound and the cell adhesion on this treated surface can be monitored online and in a label-free way by RWG biosensor [70]. C, Kinetic curves of nanoparticle penetration into cells monitored by RWG biosensor [71]. D, Kinetic curves of exosome (small extracellular vesicles) adsorption [124]. E, Signalization kinetic curves of BJAB cell line with different concentrations of BCR-specific antibodies [76]. F, 3D image of a cell by holographic transmission microscopy [49]. G, The measured population distributions of single-cell adhesivity and the corresponding lognormal fits (red lines) for the control population (left) and for the employed chondroitinase ABC (ChrABC) enzyme concentration (right) by single-cell RWG biosensor. The ChrABC enzyme digest the chains of the glycocalyx of the cells [125]. H, Subpopulation analysis of HeLa cells on different RGD densities by computer controlled micropipette (left), dependence of the fraction of weakly, moderately, and strongly adhered cells on the RGD density. Histograms of cell detachment calculated from the detachment curves received by computer controlled micropipette (right). The inset shows the poly(L-lysine)-graft-poly(ethylene glycol-RGD to) poly(L-lysine)-graft-poly(ethylene glycol) percentage ratio (Q) of the surfaces on which the cells were seeded [58]. I, Single-cell level high-throughput adhesion force and energy results of large cell population obtained by the calibrated biosensor. Adhesion spectrogram: the representation gives insight to the real-time single-cell level statistics of a typical measurement. The color bar indicates the relative frequency on a logarithmic scale of a given bin value of force, energy or integrated wavelength shift (interpolation degree k = 0). The inset on the right shows a typical force-distance curve of a measured cell and its evaluation by FluidFM [33].

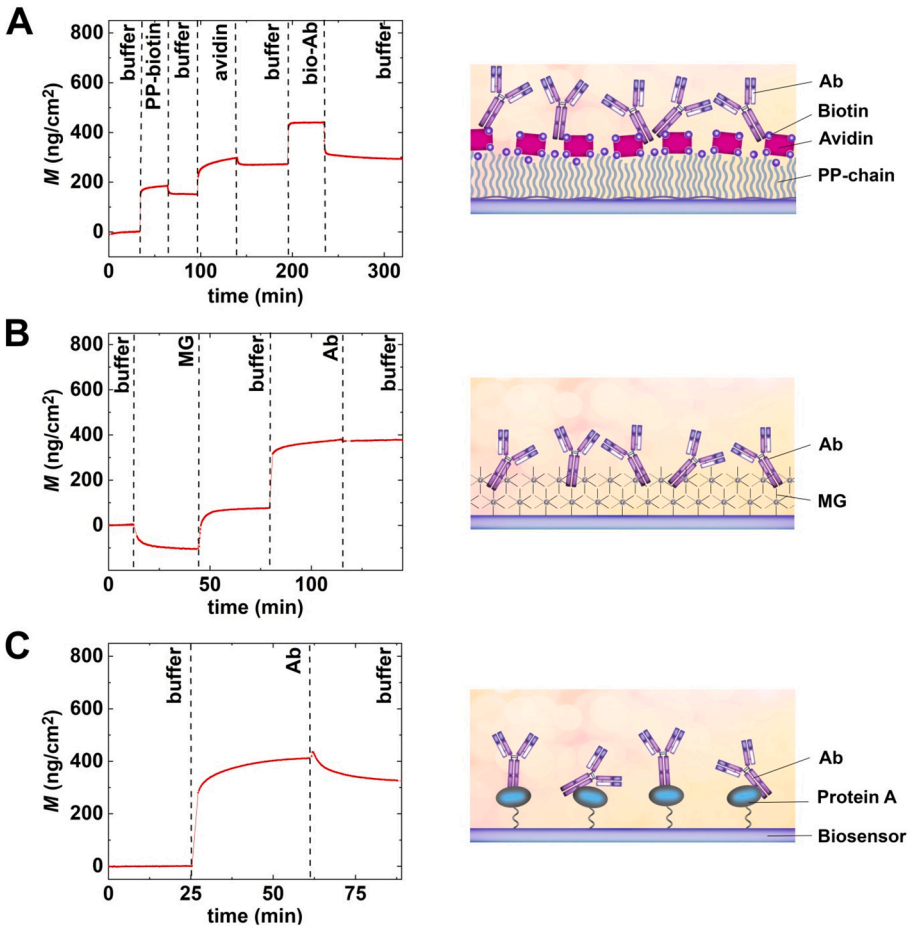

Fig. 5. Various strategies to immobilize specific antibodies on solid surfaces was performed by Farkas et al. Optical Waveguide Lightmode Spectroscopy (OWLS) was proved to be an excellent tool to characterize both the in-situ layer build-up and the orientation of the deposited antibodies. The latter was performed through optical anisotropy analysis of the fabricated thin films. Oriented layers show optical anisotropy, which is missing for a randomly oriented antibody layer. A. PLL-g-PEG-biotin avidin B. Mix&Go (also called AnteoBind) and C. protein A based coupling [147].

and stiffness for example and as well the release of captured cells for example [133].

The phenomenon of non-specific binding (NSB) is of outmost importance in biosensor measurements. During and after functionalization, biomolecules, especially from complex samples, can bind and absorb onto the non-blocked sensor surface, furthermore secondary antibodies used in many assays may bind in a non-specific manner. We recommend the review of Frutiger et al. discussing the theory of NSB [134] and also the review from Lichtenberg et al. discussing the problem from a more practical point of view [135]. Similarly cells may as well bind to the sensor surface in a nonspecific manner, both through van der Waals forces and in the case when applying organic solid supports some cell types may bind readily to the support without regard on the attached ligand [136]. The functionalization of the biosensor surface is typically based on covalent modification of the sensing surfaces. Traditional sensing surfaces are made of either metals or dielectric (e.g. metal-oxide, glass) materials. Nowadays the choice of material is shifting towards plastic materials, which are cheaper and versatile. It holds especially true for cell-based biosensors taking polystyrene from conventional cell/tissue culturing. The surface of tissue culture polystyrene (TCPS) is modified with carboxyl, hydroxyl, ketone, or formyl groups to permit cell attachment and proliferation.

The first step of covalent modification of sensing surfaces (inorganic or organic) is a promoter molecular layer, typically a silane layer. The silane molecule is characterized by head groups (e.g. alkoxysilane) for the covalent binding to the surface and end groups (amino, mercapto, carboxyl, epoxy, etc.) representing a moiety to the environment for further cross-linking. The silane modification of surfaces has been extensively studied and discussed in monographs [137]. It provides a stable cross-linking with various chemical moieties. A typical example of silane-based immobilization is the successful application of IgG proteins in biosensors, immunosensors, and microarrays [138,139]. Although silanization is a possible route for TCPS surfaces, these surfaces are usually plasma treated (etching and oxidation) converting the polystyrene into a highly carboxylated form. Other plasma processes yield a hydroxylic surface where the above described silane chemistry can be used to crosslink a ligand or a biopolymer of choice.

In cell-related biosensor studies, it was obvious to use the surface coating techniques of those explored already in cell cultures. These coatings have been developed with the aim of improving culture practice using extracellular matrix analogs, such as collagen. Other biological polymers, such as agar, poly-L-lysine, or cellulose, were also put in practice. A relatively well-understood step of cellular adhesion is related to a specific recognition of the tripeptide motif RGD by the integrins, as discussed earlier, leading to focal adhesion. Many of the biopolymers (natural or synthetic) utilize this RGD motif to create a natural environment for the cells that resembles the most to the extracellular matrix. As different adhesion molecules were discovered, the related moieties were identified [140]. These are the so-called cues that can significantly enhance the cell adhesion and thus utilized in surface coating.

Parallel investigations were run in order to understand how the surface polarity (hydrophobicity) and stiffness affect cell viability and proliferation. These surface properties were categorized to chemical and physical (mechanical); however, it turns out eventually that these are interrelated. Regarding the surface polarity, the water contact angle is used to characterize the surface quality. It was used frequently to monitor the preparation steps in simple biosensor experiments to assess the efficiency of a coating step. It was thought that water contact angle would directly determine the adsorption of proteins and consequently the cell behavior on surfaces, however, recent studies demonstrate a less

decisive role [141]. Nevertheless, it is generally accepted that a moderately hydrophilic environment is advantageous for cell adhesion. For example, mesenchymal stem cells have been shown to differentiate via adipogenic or osteogenic pathways on t-butyl and phosphate –functionalized surfaces, respectively [142]. Other findings also show that cell phenotype is related to the properties of the matrix surface. Mechanical properties [143,144], density [145] of bioactive molecules could induce macrophages polarization, and promote tissue regeneration.

The advancements in the implementation of dynamic surfaces offer a highly useful tool by enabling the modification of the substrate and the ligands applied in the biosensor. These surfaces allow changing hydrophobicity and stiffness for example and as well the release of captured cells for example [146].

4. Label-free blood cell characterization with biosensors

Cellular elements of the blood, including leukocytes, erythrocytes and thrombocytes all rise from the pluripotent hematopoietic stem cells in the bone marrow. Through multiple steps these cells differentiate into myeloid and lymphoid precursor cells. These precursors have limited differentiation potential. The myeloid lineage consists of monocytes (and their progeny the macrophages and some of the dendritic cells), dendritic cells and granulocytes. It should be noted that the dendritic cell may arise from multiple origins. The lymphoid lineage contains T and B cells and natural killer cells [148].

The cellular composition of blood determines the availability of the various cell types. Since the proportion of leukocyte subtypes is already informative the composition of blood the cells of interest measured in the assays are to be identified. Reference intervals of main leukocytes subpopulations are defined [149] and provide highly important insight when designing biosensor based measurements with the goal to study a specific subset. As discussed, briefly, the label-free techniques just reached the point of leukocyte subtype identification, and therefore the integration of these in biosensor platform is highly desirable. Until these are widely available classical stains and post measurement label-based identification is to be considered/ recommended.

The cells of the innate and adaptive parts of the immune system orchestrate the defense against foreign antigens. To mount a sufficient immune response, immune cells are known for their ability to produce, recognize, and respond to cytokines. This small protein, polypeptide agents are the primary means for communication between immune cells throughout the body. These immunomodulatory agents may provide signals in an autocrine or paracrine fashion, and orchestrate the humoral and cell based immune response. Importantly the effect of cytokines is pleiotropic; therefore, their measurement requires multiplex approach to determine the direction of the immune response. In a simplified view pro- and anti-inflammatory cytokines are distinguished; however, their role in controlling differentiation, signaling, activation, and proliferation for example is more and more understood.

The heterogeneity observed in leukocyte responsiveness to various stimuli even in genetically identical cells, due to the stochasticity of the biochemical reactions controlling their phenotype, the net effect of cytokine induced response in each cell type is hardly deductible from the cytokine profiles in various patients. An important aspect, when studying immune cell response, is that multiple cell subsets may play a role in a given immune status; therefore, it is recommended to study their responses from a systems point of view to various stimuli [150]. For immune monitoring purposes the current guidelines recommend avoiding preprocessing of blood to minimize preanalytical error [151]. This could possibly help us minimize intra-center variability and as well provide the foundations of POC applications. In order to handle the heterogeneity, the application of systems approach is beneficial just as in case it was demonstrated with gene expression profiling in the case of investigating Toll like receptor TLR ligands for example [152].

Single-cell parameters may not alleviate the burden of clinical studies aiming to identify cellular behavior and activation in healthy and patients group. However, biosensor-based cell characterization may lower reagent costs and may allow simplified diagnostic approaches. Defining normal in a diverse population is a highly difficult task and it is a prerequisite of applying biosensor-based cell characterization in healthcare. Therefore, establishment of the baseline reference intervals is to be done for each parameter. Despite defining underlying mechanisms in various states of the human immune system, the question “How is my immune system?” is still waiting to be answered, no simple readout ready yet to give an overall response [153]. When studying the state of the immune system and its reaction to perturbations, such as vaccination, both genetic and phenotypic variation is to be taken into consideration to provide the means of precision medicine, in order to predict immune response quality based on biomarkers [154]. Similarly identification of baseline level of biomarkers is an important factor in predicting therapy response in tumor immunology [155] and organ transplantation [156]. Multiple layers of heterogeneity can be identified within immune cells. First the subpopulations of the leukocytes, that can be more distinct, such as in the case of T or B cells, or more continuous as in the case of macrophage polarization for example. The second layer of heterogeneity is the responsiveness and hence the activation of the cells. This important aspect is understood to be multifactorial, age, sex and genetics all play a role on a transcriptional level [157]. Furthermore, non-heritable factors, such as previous infections, as in the case of cytomegalovirus, may explain a large portion of the observed variation [158].

In the following part we will discuss the most important achievements regarding monitoring leukocyte activation with biosensors. Fig. 6 shows examples of leukocyte functions detectable by label-free methods.

4.1. *Neutrophil granulocytes*

The name granulocyte refers to the densely granulated cytoplasm of these cells. Subgroups of the granulocytes are differentiated on the staining properties of these granules: neutrophil, eosinophil, and basophil granulocytes. Each of these granules contains various effector molecules, characteristic to the cell type. Due to their oddly-shaped nucleus, they are also often referred to as polymorphonuclear cells (PMNs). These cells are short lived, and their numbers may rise in the circulation due to infection for example. Under normal circumstances only fully matured granulocytes leave the bone marrow, however, during infection precursors also appear in blood samples.

After entering the circulation, neutrophils migrate to inflamed or infected tissues where they play an important role as the first line of the cellular immune response. Classical neutrophil functions include the release of antimicrobial granule content, phagocytosis, and killing of microbes through the production of reactive oxygen species and ejecting their nuclear material to trap microbes in a process called NETosis [159]. However, recent research showed that neutrophils are transcriptionally active, produce various factors, such as cytokines and other immune mediators, and are thus important in shaping the immune response as well. Moreover, recent research showed that neutrophils themselves are a heterogeneous population. Their maturation is completed in the circulation, during which their receptor expression profile may change for example due to the presence of various foreign molecules. Importantly their functional capacity also seems to be affected by the circadian rhythm. Lymphocyte- neutrophil ratio was also found to be a useful predictive marker of tumor progression. Neutrophils express various pattern recognition receptors, Fc and complement receptors as well [160]. During sepsis, a life-threatening condition, many neutrophil functions, such as tissue migration, egress from the bone marrow, and NETosis capacity, were found to be impaired. It is also suggested that restoring neutrophil functions during sepsis facilitates the clearance of bacteria and restoring homeostasis [161]. The constant presence of microbes activating the immune system leads to hyper-inflammation, leading to a cytokine storm. Importantly biomarkers

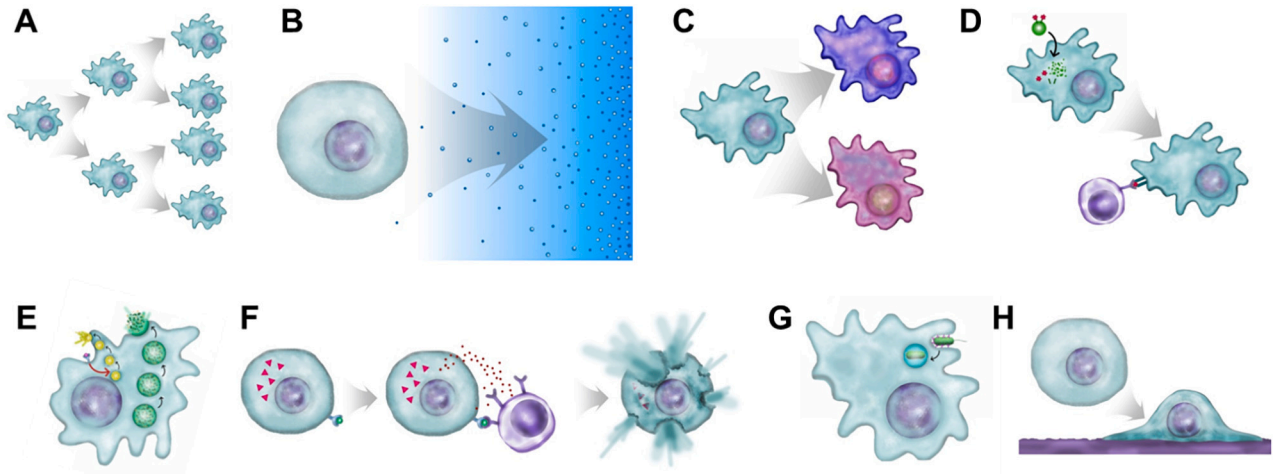

Fig. 6. Examples of leukocyte functions detectable by label-free biosensors A. proliferation B. chemotaxis C cell polarization and activation D. antigen presentation E. degranulation and secretion F. cytotoxicity G. phagocytosis and H. adhesion.

available for early detection of sepsis in the clinics are limited and affected by the heterogeneity of the patient population and the time sensitive nature of biomarker elevation [162,163]. Despite being a major cause of death for patients in intensive care units, there is no gold standard assay to monitor sepsis and new diagnostic and prognostic biomarkers are needed to characterize sepsis [164]. Due to the complex nature of sepsis, a personalized approach is recommended. Although in sepsis the impaired chemotaxis of neutrophils contribute to insufficient elimination of microbes, in other inflammatory diseases increased chemotaxis is suspected to contribute to the development of the disease, such as in the case of atherosclerosis. Chemotaxis is performed classically in a Boyden chamber, where a porous membrane allows the cell to migrate only actively to the bottom chamber containing attractants. Using impedance-based adhesion assays after coating the bottom of the membrane with fibrinogen and poly-L-lysine of a cell invasion and migration plate Cano et al. designed an Real-Time Cell Analysis (RTCA) assay that allows label-free monitoring of neutrophil chemotaxis driven by leukotriene B4 and IL-8 [165]. However, when studying neutrophil migration further parameters are to be studies as well such as velocity and directedness [166]. In their recent work, Jeon et al. showed that solely based on their intrinsic electrical properties, leukocytes from healthy donors and patients with sepsis can be distinguished with high precision [167]. On the basis of their isoelectric position, activated and nonactivated cells were identified. After treatment with phorbol 12-myristate 13-acetate (PMA), activated cells showed a lower isodielectric point this application offers a great step towards the biosensor-based sepsis identification, however, the sample size is to be increased, and differential disease cohorts are to be included to further assess the potential of the method. Ellett et al. identified various aspects of neutrophil motility in a polydimethylsiloxane (PDMS) microfluidic system to be strong indicators of sepsis [168]. CD64 expression on neutrophils is a pro-inflammatory biomarker. Hassan et al. designed a microfluidic system with a capture chamber and counted the entering and exiting cells. They found that when combined with classical methods to diagnose sepsis, this measurement can increase the area under the curve of the receiver operating characteristic curve in a clinical setting, and therefore shows potential as a POC device for sepsis patient stratification [169]. Similarly, neutrophil rolling in *E*-selectin coated PDMS showed characteristic alterations in the blood samples from patients with diabetes mellitus compared to the control group, importantly, neutrophil rolling speed was reported as a functional neutrophil biomarker, indicating low-grade inflammation, next to lower neutrophil counts [170]. For a similar purpose, Petchakup et al. developed a PDMS based microfluidic impedance cytometer that provides an integrated solution for prepurification and functional characterization via detecting dielectric properties, opacity and cell size, and thus determination of neutrophil extracellular trap formation [171]. These methods could prove to be a label-free alternative to current methods applied and developed for NET quantification [172]. In their microwell-based localized surface plasmon resonance based setup Ali et al. demonstrated the label-free analysis of single neutrophil granulocytes following PMA treatment [173]. Deep-learning based image analysis based on bright-field microscopic images Hhaung et al. were able to classify activated and inactivated neutrophils, with further improvement these efforts could prove to be useful later in image based WBC classification as well [174]. Christensen et al. showed that refractometric resonant wavelength guide-based measurements can be used to detect Formyl peptide receptor agonists based on neutrophil activation by measuring dynamic mass redistribution of cells sedimentated on the sensor surface [77]. Similarly, Bunnfors et al. studied [175] nanoparticle based activation of neutrophils with a label-free capacitive sensor, however these sensors are not yet able to monitor single cell responses. Arend et al. were able to differentiate between bacterial and fungal sources of infection with the application of Raman spectroscopy. However, this method applies fixed samples [176]. To study the interaction of live bacteria with neutrophils microwell-based chambers were designed, however, to our understanding such platforms were not yet combined with biosensors [177]. Finally, Ekpenyong et al. determined further mechanical and morphological parameters of neutrophil activation, such as increased deformability, aspect ratio, and circularity [178].

4.2. *Eosinophil, basophils granulocytes and mast cells*

Due to their shared role in allergic, asthmatic and inflammatory reactions eosinophil and basophil granulocytes are often discussed together [179]. The number of these cells in the circulation is normally very low, below 3% for eosinophils and below 1% for basophils, while mast cells are only found in tissues. These cells also share the common appearance of Fc epsilon receptors [180]. The high affinity receptors for IgE, when cross-linked mediate the instant activation and degranulation of the cells, which mirrors and explains the symptoms of allergic reactions. Interestingly, FcεRI expression is induced by high levels of serum IgE, most likely due to IgE stabilizing the FcεRI in the membrane [181]. These cells are also transcriptionally active and their contribution to shaping the immune response is also highly important. Although the number of basophils and eosinophils is low in the circulation, it can rise quickly at sites of tissue injuries, parasitic infection and allergic reactions.

Allergic diseases are caused by a hypersensitive immune response to,

in most cases, otherwise harmless environmental antigens, also known as allergens. Allergic reactions may affect both the respiratory and the digestive systems. Most allergic reactions are triggered by the presence of allergen specific IgE molecules. The high-affinity IgE receptor (FcεRI) can bind monomer IgE, so these molecules are readily present on the surface of cells expressing FcεRI. This process is also called cell sensitization. Therefore when allergens get in contact and bind to the FcεRI bound IgE molecules, they can directly crosslink these receptors, resulting in their oligomerization and thus trigger immediate cell activation [182]. Basophils and Mast cells are the main effectors of allergic reactions. Despite their functional similarity and shared ontogeny, their transcriptional regulation, function, receptor expression, involvement in inflammatory diseases [183], and their set of inflammatory mediators differs as well [184,185].

Current methods of testing allergens focus on the detection of allergen specific serum IgE or apply skin prick tests to verify the allergens. The results of these tests are not predictive enough, moreover, in some cases the provocation may be potentially dangerous (triggering anaphylaxis). Therefore, a new line of diagnostic assays has been developed aiming to monitor basophil activation itself through the detection of cell associated biomarkers using flow cytometry [186]. These in vitro basophil activation test approaches alleviate the burden of potentially life-threatening allergen challenges and may be less limited by allergen availability [187]. Such approaches based on cell surface marker expression were successfully implemented to monitor oral immunotherapy efficacy in food allergy [188]. Importantly from the point of the view of the development of label-free approaches, degranulation of Basophils also triggers morphological changes [189].These cells highly express FcεRI, and when cross-linked through IgE and allergens, their activation results in the release and de novo synthesis of inflammatory mediators, such as histamine, lipid mediators, cytokines and other inflammatory mediators. However, to a lower extent, IgE-independent activation of basophils and mast cells is known as well, for example by PAMPs and cytokines. Through the cytokine secretion, they also contribute to the shaping of immune response, for example, through the polarizing T-cell differentiation and recruitment of eosinophils to the site of activation [190,191].

Eosinophil granules contain mediators of the parasite-specific immune response. These granules contain peroxidase, ribonuclease, lipase, plasminogen and major basic protein, and are released in a process called degranulation [192]. Granules can be released through multiple mechanisms, classical degranulation, cytolysis, and piecemeal degranulation [193], a process that allows the selective release of inflammatory mediators and cytokines, for example, IL-4 [194]. Through cytolysis intact granules are released from the eosinophils which can be found in tissues with eosinophilic activation, and express cytokine receptors and are able to release their cargo upon activation [195]. These mediators are toxic to both host and parasite tissues. Eosinophils are also capable of the formation of extracellular traps which consists mitochondrial DNA and secretory granules [196,197]. Although their contribution to fungal, viral, bacterial and, most importantly, parasitic infections have been shown, their role remains highly enigmatic. Their role in the pathogenesis of asthmatic diseases is the most studied [192]. Hypereosinophilic syndrome is characterized by highly elevated numbers of eosinophils, with evidence of tissue infiltration of eosinophils and extracellular deposition of eosinophil-derived proteins in the affected tissues. The current treatment approach for these patients is to minimize tissue injury in an eosinophil-specific manner. This selective inhibition of the eosinophil may also contribute to the improved understanding of its role in homeostatic processes [198]. In a novel approach, eosinophils were targeted with a monoclonal antibody specific to the alpha chain of the IL-5 receptor. Surprisingly this treatment lead to almost complete depletion of eosinophil granulocytes, without any side effect in the short term [199], however, long-term complications cannot be excluded as of now [200]. This clearly show that eosinophils are evolutionary conserved, likely due to their role in fighting parasitic, such as helminth infections. However, their role in healthy in normal immune response seems redundant [201]. Unlike basophils and mast cells, eosinophils only express a small amount of FcεRI and eosinophils are neither activated IgE-mediated receptor crosslinking nor could bound monomer IgE [202]. However, this seems inducible since eosinophils of patients with hypereosinophilic syndrome shows clear contribution of FcεRI in helminth specific response [203]. The contribution of eosinophils is widely studied in respiratory diseases such as eosinophilic asthma [204], chronic lung disease – where eosinophil content of the sputum is suggested to be a predictive factor for responsiveness to corticosteroid treatment [205], and eosinophilic esophagitis for example [206]. However, the classification and diagnosis of eosinophilic disorders require further improvement [207].

Eosinophils from patients with eosinophilic asthma, rhinitis and atopic dermatitis showed that the granule content and morphology of their circulating eosinophils based on transmission electron microscopy is comparable to those from healthy individuals [208]. Importantly, these eosinophils were fixed directly after venous blood sampling. In a similar study in which healthy and acute asthma donors' eosinophils were allowed to spread on glass at 37 °C (where eosinophils likely activate by adhesion) a marked difference was observed in their spreading and morphology when measured by AFM [209]. Although this conclusion was based on very few samples, this approach highlights the need to study cells in a functional setting. However, based on optical diffraction tomography, Kim et al. showed that the density of the eosinophil granules is higher in asthma patients. This technology also allows for the monitoring of changes in the refractive index of individual organelles in a three dimensional distribution [210]. Image analysis based on imaging cytometry also allows for the label-free detection of activation state of individual eosinophils. Using the transmission and light scattering information Piasecka et al. showed that the activation state of eosinophils can be characterized, offering a diagnostic alternative to biopsy based evaluation [211].

Various methods have been introduced to test mast cell degranulation, as a model for IgE- and allergen-mediated cell activation. RBL—2H3, a basophilic rat cell line, is widely used in these experiments to study inflammatory processes resulting in histamine release. Imaging SPR-based studies by Hide et al. established not only the mast cell based studies, but showed that the technique is applicable to detect cellular response next to molecular interactions. In their first paper they showed that the addition of allergen to sensitized mast cells triggers a dose dependent response of the cells that is not affected by the exocytosis of the granules. The activation was inhibited in the presence of various inhibitors as expected [212]. Subsequently, the application of this system was expanded to other cell types, primary basophils [213] and serum testing using a modified RBL-2H3 clone expressing human IgE receptor (FcεRI) [214], optical fiber SPR based detection [215], later adapted to impedance-based assays [216], and most importantly to single cells [217]. Most recently with a combined impedance and SPR based measurement, they showed that results of the combined measurements show similar outcomes and activation of the cells [218]. Abassi et al. showed that crosslinking IgE sensitized FcεRI with specific antigens resulted in characteristic response measurable in impedance electrode based assays and the activation and resulting cell index showed response to well-known inhibitors [219]. Following this line of experimentation, an impedance-based assay was applied to study the effect of various inhibitors of degranulation signaling by evaluating the time - dependent cell response profiles resulting from these experiments. The response curve and the generated cell indexes allowed clustering of the 145 inhibitors test using the ECIS RTCA system, and clearly show that the response is dependent on the activation of the various signaling molecules' phosphorylation. Furthermore, the setup was also able to detect apoptosis of the cells [220]. Examples of label-free characterization of granulocytes are shown in Fig. 7.

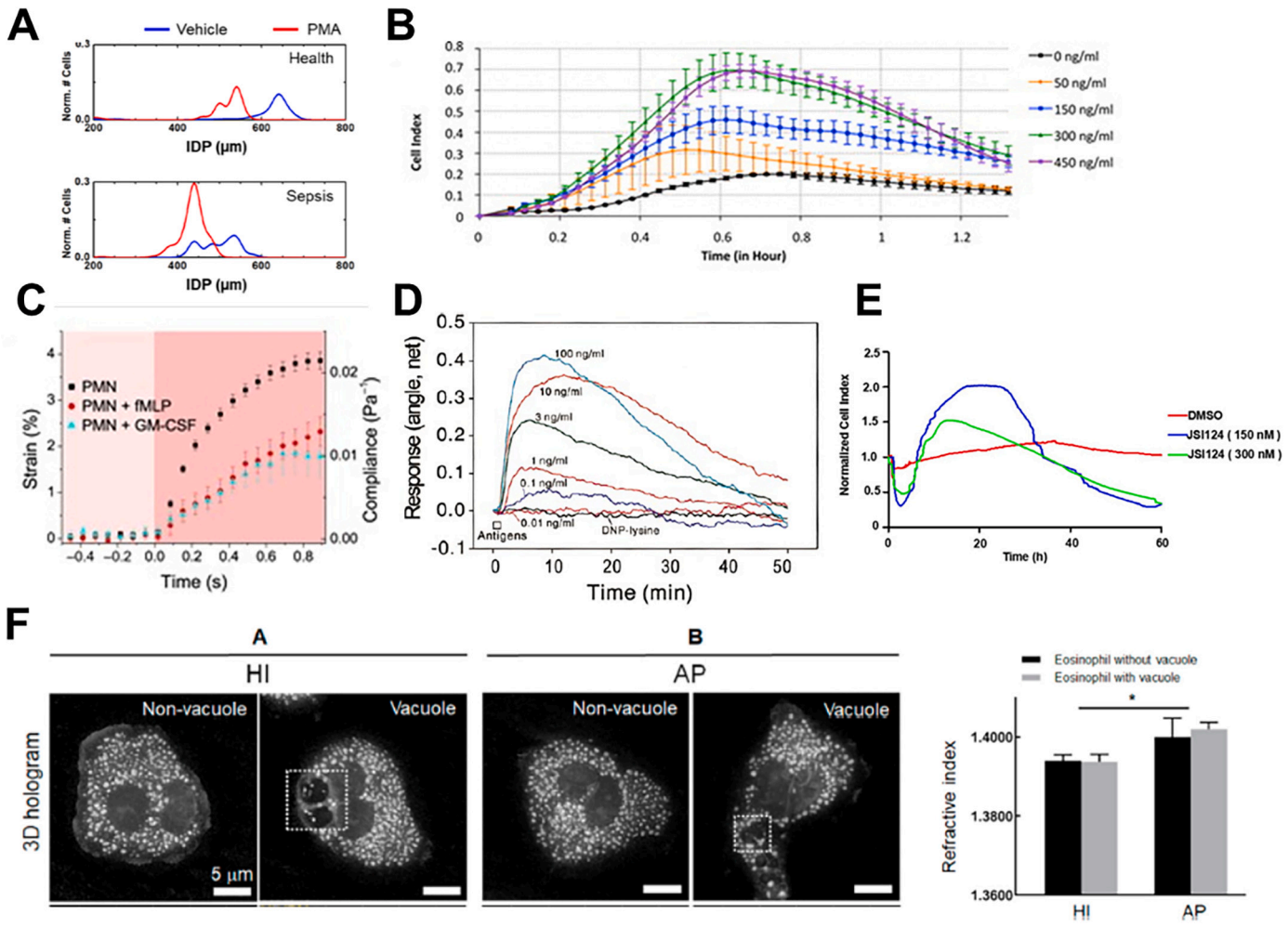

Fig. 7. Examples of label-free characterization of granulocytes. A, isoelectric position distribution in healthy and sepsis patient donors' neutrophil granulocytes [167]. B, dose dependent kinetic effect of IL-8 on neutrophil chemotaxis [165]. C, neutrophil activation results reduced deformability [178]. D, Dose dependent activation of basophilic cell line RBL-2H3 in SPR [212]. E, Dose dependent apoptosis of basophilic cell line RBL-2H3 based on impedance measurements [220]. F, Optical diffraction tomographic pictures of healthy and asthma patient individuals. Granules in asthma patients show elevated refractive index [210].

4.3. Monocytes, macrophages and dendritic cells

Cells of the mononuclear phagocyte system (MPS) found in blood include monocytes, macrophages, and dendritic cells. These cells develop from the macrophage and DC progenitors (MDP). Monocytes may differentiate into macrophages upon migrating into tissues, and monocyte derived DCs as well. However, MDPs give rise common progenitor of human DCs (CDP) that will eventually differentiate into conventional dendritic cells (cDCs) [221,222] on the other hand, plasmacytoid DCs are mainly of lymphoid origin [223,224]. The precursors of all dendritic cell subsets can be found in the circulation as well [225–227]. This complicated ontogeny also calls for a novel nomenclature that includes cellular development to categorize these cells as recently suggested [228]. The common characteristic of these cells is their phagocytic potential. Through pattern recognition [229,230], Fc [231] or complement receptors [232] they contribute to the clearance of both apoptotic cell debris and killing of invading microbes. MPS cells are highly diverse, as illustrated by their ever-evolving classification [233]. Monocytes were initially classified according to their expression of CD14 and CD16 cell surface receptors [234]. Classical monocytes give rise to intermediate and non-classical monocytes in the circulation. However, the function of these subgroups was found to overlap in many cases. Subsequently, this classification was refined further and novel subgroups of monocytes were identified by flow cytometry [226], and with the transcriptomics based on gene expression profiling, additional subpopulations were found [235–237]. Similarly, DCs are highly heterogeneous, with now 5 subsets of cDCs suggested, however, some of the suggested subpopulations might as well be precursors of other subgroups [224]. Tissue resident macrophages seem to be a distinct self-maintaining populations with minimal contribution of blood monocytes [238–240]. During inflammation after extravasation monocytes and their progeny contribute to the pro-inflammatory response later on monocyte derived macrophages may contribute to tissue remodeling and wound healing that when not tightly controlled can play a role in pathologic conditions as well [241]. This is especially important in for example healing after various implants, how various parameters of the implant such as stiffness, topography, geometry, etc. shape macrophage polarization and facilitate regulators (M2) macrophages over the inflammatory macrophages(M1) [242]. The polarization of macrophages is controlled by the nature of the pathogen, the available cytokines, and tissue environment, resulting in a great diversity among cells of the MPS, especially within macrophages [233]. The role and tissue distribution of pDCs, cDCs and monocyte-derived dendritic cells differ as well [224,225].Just as importantly after phagocytosis these cells bridge innate and adaptive immunity through antigen presentation, and thereby in the selection and priming of T lymphocytes, also by producing cytokines and chemokines to control the direction of the immune response. Therefore these cells are of outmost importance in

autoimmune diseases [243], tumor immunology [244–246], and infectious diseases [247–249], among others.

The spreading of monocytes was demonstrated with OWLS on glassy silica–titania sensor surfaces. Here, adhesion and spreading was inhabitable by increasing serum concentration. This can work the other way as well, for example serum inhibition can be used to block aspecific binding of proteins [250].As a follow up various other label-free techniques were applied to study adhesive properties of monocytes, monocyte derived macrophages (MDM) and dendritic cells on fibrinogen and PLL-g-PEG–RGD coated surfaces. All cell types investigated here showed the strongest binding to fibrinogen followed by the RGD containing surfaces, and no binding on the PLL-g-PEG control surfaces when measured with optical label-free biosensors. The adhesion of DCs resulted in the highest wavelength shift closely followed by the MDMs, with monocyte binding showing a weaker adhesion. The authors found that these results were in agreement with computer controlled micropipette based results, indicating that an increased wavelength shift also means a stronger binding to the surface. These results were found to be in agreement with other classical end-point based methods [251]. These important results show that interactions of cells with components of the extracellular matrix (and potentially any molecule of interest) can be monitored in a label-free manner.

The adhesion of monocytes mediated by Fc receptors to IgG subclasses and immune complexes was investigated by SPR imaging. Immune complexes were generated on the surface of the SPR sensor by amine coupling of citrullinated RA diagnostic peptide dendrimers and immunoglobulins, and BSA as a negative control. After blocking, serum samples were incubated and immune complexes generated on the chip were detected using U937 cell line [252] -most likely this adhesion is mediated by Fc receptors. Although the experiment was not performed on cells of the MPS the role of Fc receptors on whole cell adhesion was presented by Temming et al. Using imaging SPR the glycan sensitivity of FcγR-IgG interaction was investigated. HEK cells, transfected with various Fc receptors, were injected into the SPR flow chamber and after sedimentation their retention rate was measured after washing with increasing flow speed [53]. Similarly, Yan et al. showed binding of bovine macrophages to rabbit IgG in QCM measurements and suggested that the presented method was capable of semiquantitatively determining Fc receptor numbers per macrophage [253].The phagocytic activity of DH82 macrophages was investigated in a QCM setup. Using zymosan as a model for particulate antigen and single walled carbon nanotubes, they investigated the phagocytic response and toxicity towards these compounds. Toxicity was determined based on cell loss as measured through the decreasing signal in QCM. The authors suggest that based on these results, QCM is a sensitive method to study cell toxicity and phagocytosis [254]. In line with these results, Dewilde et al. showed that sodium-azide-triggered apoptosis can be monitored. They also found that the QCM based signal allows earlier detection of the cellular response, even before morphological changes become apparent [255]. Not only cell-substrate but cell-cell interactions can be studied using label-free methods. After activating endothelial cells by LPS their interaction with the substrate was monitored in real time using impedance-based real-time cell electronic sensor system. In the presence of U937 cells endothelial cells showed reduced adhesiveness to the gold electrodes, indicating that human umbilical vein endothelial cells, due to the presence of monocytes, change their phenotype to enable increased leukocyte infiltration [92].

Dendritic cells can as well be applied to detect bacteria. *E. coli* strain K12 and its LPS mutants were tested to determine how the different LPS forms affect NO production in DCs and macrophages. Although the detection here was not biosensor-based, the results clearly show that the DC activating properties of various compounds can be exploited in biosensor-based measurements, just as the authors suggest [256]. Similarly, amperometric detection allowed selective detection of H_2O_2 in response to TLR ligands, allowing the kinetic detection of the triggered cell response [257]. PMA activation of macrophages lead to a similar result when investigated in a PDMS based microfluidic system in combination with a HRP/PEG hydrogel/ Au electrode based electrochemical sensing system [258]. LPS macrophage interactions were as well studied using the Ana-1 cell line in electrochemical impedance assays. The dose dependent cytotoxic effect of LPS was demonstrated as a method to reliably detect the morphological changes. In an innovative step cell attachment to the sensor surface was carried out by internalization of magnetic particles by macrophages on the magnetic glass carbon electrode [259]. Using Fourier transform infrared spectroscopy, Veiseh et al. demonstrated that LPS induced macrophage activation results in a shift in the detected infrared spectrum and therefore potentially enables identification of infected macrophages and other activating compounds as well [260]. Cao et al. showed that macrophage activation triggered by cytokines is also detectable using impedance-based detection using real time cell analyzer. The working scheme applies gold electrodes incorporated into microtiter plates. LPS and IFN-γ showed a dose and cell number dependent effect on the cell index and this response was dampened in the presence of various inhibitors [261]. Quantitative phase imaging combined with Raman spectroscopy produces complementary datasets allowing the determination of macrophage activation status in response to LPS and the effect of the inhibitor applied. Since individual cells can be characterized with this approach, cellular heterogeneity can be determined [262]. Bertani et al. successfully applied hyperspectral reflectance confocal microscopy to analyze macrophage polarization on glass coverslips. The principal component analysis resulted in an accuracy over 98% in the classification [263]. In their monocytes with internalized pathogens (MIP) assay platform, Liao et al. successfully sorted infected monocytes based on their increased stiffness and size. This platform allows fast detection and separation of monocytes infected with blood-borne pathogens [264]. Examples of label-free characterization of mononuclear phagocytes are shown in Fig. 8.

4.4. Lymphocytes

T and B cells belong to the adaptive arm of immunity and, therefore, have the ability to recognize foreign compounds whose recognition is not encoded in the germline. These cells are capable of mediating acquired immune response by recognizing novel antigens. The diversity of antigen recognizing elements T and B cell receptors (TCR and BCR) is the result of the VDJ recombination, a process of somatic recombination only known in the T and B lymphocytes. For B cells, this process takes place in the bone marrow and results in the naive B cell repertoire [265].

Naive T cells arise from the differentiation of CLPs in the bone marrow. The naive repertoire is shaped through multiple steps of negative and positive selection, which ensures that the naive T cells bind strongly enough to MHC I or MHC II molecules and have a functional CD4 or CD8 coreceptor, and, on the other hand, they do not react to MHC complexes with self-peptides. The selection process results the two main T-cell subsets: CD4+ T helper (Th) and CD8+ cytotoxic T cells (Tc). Th cells recognize peptide-MHCII complexes on B cells and macrophages and other professional APCs, and this interaction leads to cytokine production by the Th cells, fine tuning, and polarizing the immune response. Cytotoxic T-cells recognizing any foreign/altered self-peptide MHC I complex triggers its cytotoxic activity and by releasing most importantly perforin and granzymes will eventually trigger the apoptosis of the target cell [266]. MHC multimers became a valuable tool and are widely used to monitor the presence of antigen-specific T cell clones [267].

While T cells are responsible for cellular immunity B cells' primary role is to provide humoral immunity. This is due to the fact that, activated B cells produce antibodies. Upon activation, they differentiate into plasmablasts or antibody-secreting plasma cells.

For further important steps from changing isotypes to affinity maturation memory formation and b cell subpopulations we redirect the reader to other reviews [268,269]. B cells develop in the bone marrow

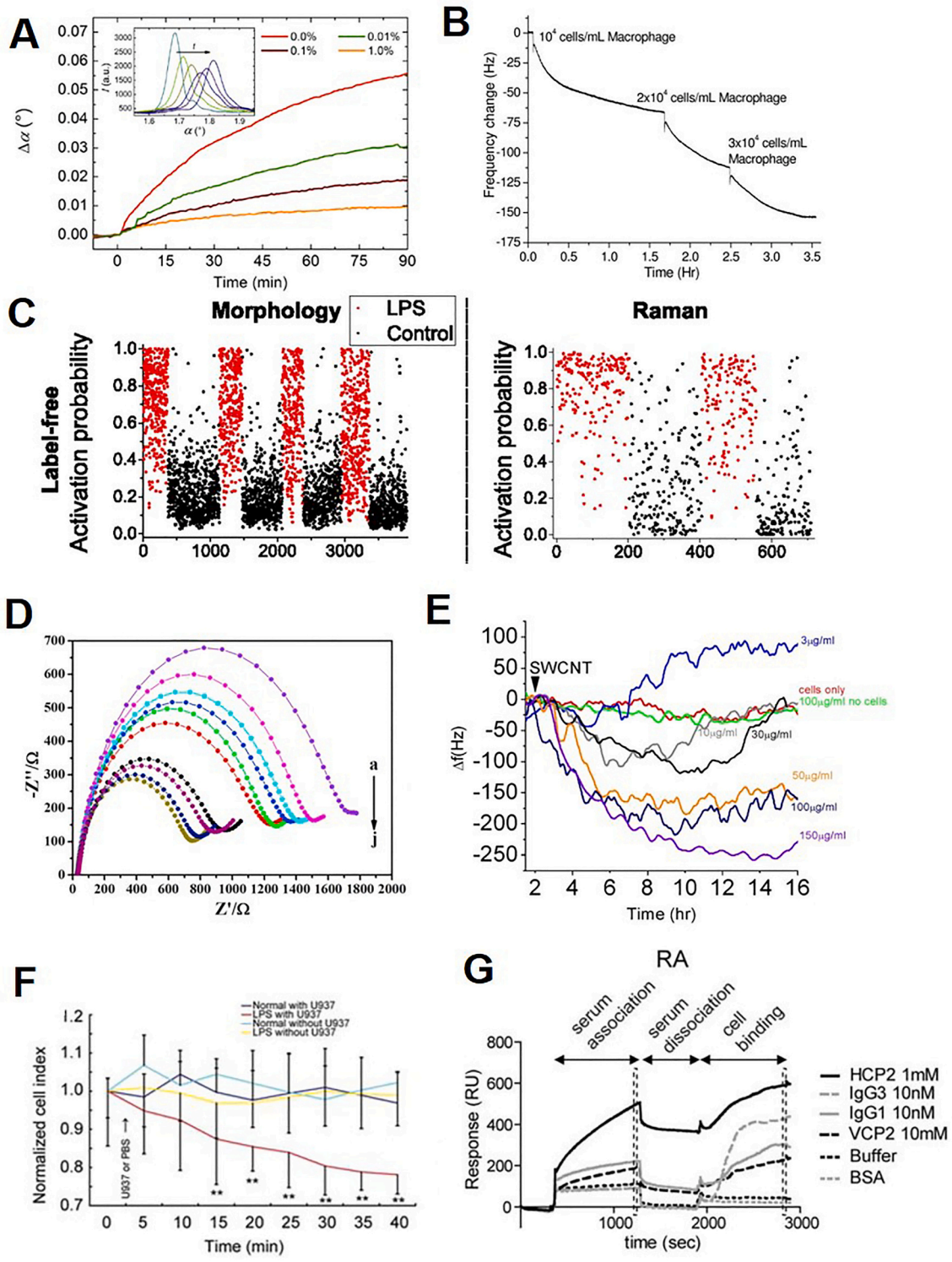

Fig. 8. Examples of label-free characterization of mononuclear phagocytes. A serum addition blocks monocyte adhesion to OWLS sensor [250]. B QCM based detection of macrophage adhesion [253]. C PHI and Raman based determination of macrophage activation [262]. D Dose dependent cytotoxic effect of LPS on u937 monocytes based on impedance assays [259]. E Dose dependent cytotoxic effect of single wall carbon nanotubes on macrophages detected by QCM [254]. F LPS induced increased permeability of endothelial cells in the presence of U937 monocytes as measured in impedance assay [92]. G U937 cell binding to control IgGs and immune complexes generated on SPR sensor surface [252].

from CLPs. In addition to interaction with other cells, BCR, the inhibitory Fcγ receptor(CD32B [270]), complement receptors [271] and pattern recognition receptors [272] play an especially important role in B-cell activation. Activation through BCR also results morphological changes of their B cells through cytoskeleton reorganization, and this feature can be exploited to detect their activation [273]. An important feature of adaptive immunity is memory formation. Both T and B cell activation results proliferation, and some of these cells will form memory cells. In the case of B cells, this activation also results the rise of long-lived antibody secreting plasma cells [274,275]. Regarding the T and B

cell content of blood cells from all stages of differentiation can be found in the blood: immature naive memory and plasma blasts as well [276]. Natural killer (NK) cells constitute the third lineage of lymphocytes. Unlike other lymphocytes they recognize and kill cells lacking MHCI or expressing FAS ligand, moreover they are important mediators of antibody dependent cellular cytotoxicity as well. These cells are important in the process of eliminating virus infected and tumor cells [277,278].

B-cell activation can be detected using RWG based methods too. Both cell lines and primary cell activation can be monitored through the acquisition of kinetic data in these systems. The activating or inhibitory responses of various compounds can be determined by measuring the DMR in these systems [75,76,78]. While these approaches do not allow single cell measurements yet, the resolution of similar RWG based measurements shows that single-cell analysis is also available for these measurements [33]. Saitakis et al. investigated the binding of HLA-A2 positive B-lymphoblast cells to HLA-A2 specific antibodies immobilized on the sensor surface. They compared acoustic-wave biosensor and SPR based measurements, and saw binding only in case of the acoustic method. While they speculated that it might to due to experimental setup, it has been clearly showed since that such approaches are suitable for immunocapture as well [108]. In a similar experiment Shanehbandi et al. demonstrated SPR based immunocapture of CD20+ B-cells using pure gold SPR sensors functionalized with CD20 specific antibody immobilized in the sensor using 11-mercaptoundecanoic acid and staphylococcus protein A. This study confirmed that both strategies can be successfully applied and even monoclonal antibodies can be screen based on cell binding [279]. The binding of CD20 specific antibody to B-cells was also demonstrated using QCM, allowing kinetic readout [280]. By combining of quantitative phase imaging (QPI) and machine learning Ayyappan et al. demonstrated that not only single B-cell acute lymphoblastic leukemia cells can be identified but disease progression can be monitored as well with this method, paving the way for large clinical studies [281]. As a next step Paidi et al. demonstrated that Raman spectroscopy based biomolecular data can add further detail to the imaging based morphological classification. Although the Raman and QPI data were not yet generated from the same cells, the authors are working on combined measurements, which could prove to be an excellent tool for the characterization of other cell types as well [282]. B-cell lymphomas can be detected using idiotypic peptides recognizing the BCR of the malignant B-cell clone. However this approach requires the prior knowledge regarding BCR specificity, resulting in a complicated workflow [283]. Antibody secretion of plasma cells is a highly researched area, since automation and simplification of identification and production of antigen-specific antibodies is highly desired. Various solutions to tackle this highly difficult task are discussed in detail elsewhere [284–288].

Few examples of how SPR can be applied to detect antigen specific antibodies have been presented as well. SPR imaging was also demonstrated as an existing tool to monitor antigen-specific antibody production of B-cell hybridomas. Milgram et al. coupled the antigen hen egg lysozyme onto the sensor surface in a multiplex manner also at different concentrations and showed that the antibody production can be determined in this system. Importantly, such flexible systems allow monitoring of other immune cells in a multiplex and kinetic manner, they note [289]. Stojanovic et al. demonstrated that antibody production of a hybridoma cell line can be detected and quantified [290]. In their theoretical work, they showed that 99% of the produced antibody can be detected on the sensor surface [291]. Abali et al. designed a microwell based inlet for the imaging SPR-based setup that allows seeding and isolation of single B-cell clones and allows the isolated measurement of their antibody production [56].

T cell activation has gained great attention in label-free biosensor studies. Lymphocyte activation results cytoskeleton reorganization and thus decreased nucleus:cytoplasm ratio, morphological and metabolic changes and these parameters as demonstrated by the following examples can be detected with various methods. T-cell activation can be detected by the increased metabolism of the cells resulting rapid extracellular acidification when activated by peptide-MHC complexes (pMHC) or by applying anti-CD3 antibodies. As demonstrated by Stern et al. this can be detected by complementary metal-oxide semiconductor based methods [292].This method, however, does not allow identification of single cells. One of the first examples in this field showed that adhesion through activating antibodies results cell spreading on the sensor surface, that is readily detectable by reflectometric interference spectroscopy in a label-free manner [293].

When compared by diffraction tomography allowing three dimensional reconstruction of individual cells Yoon et al. identified multiple features, such as sphericity, cell volume, dry mass and cell surface, that distinguish lymphocytes from macrophages. Moreover, the fine resolution provided by this technique has the potential to identify morphological features to study leukocyte activation in a clinical setting [294]. This technique, when combined with machine learning, even allows classification of non-activated lymphocyte subsets, a difficult problem other label-free techniques not yet capable of [295]. Using the same technique combined with deep learning algorithms immunological synapse can be studied as well in detail in a dynamic manner allowing evaluation of T-cells with chimeric antigen receptor [296]. Fluorescent lifetime imaging of FAD and NAD(*P*)H was applied to determine T-cell subsets and their activation state. Cell activation was performed using tetrameric antibodies against CD2, CD3, CD28. When compared to quiescent non-activated cells, cell size and redox ratio allowed for identification with high precision when compared to classical flow cytometry based evaluation [297]. Using the same dataset Wang et al. showed that image analysis by convolutional neural networks based on the NAD(*P*)H autofluorescence images alone results similar accuracy [298]. Using similar stimulation, Guan et al. identified activated T cells in impedance-based assays, importantly, they also demonstrate that the activation can be controlled using various inhibitors as well [299]. Single-cell acoustic force microscopy allows for label-free determination of cellular avidity to various compounds. T-lymphocyte adhesion on fibrinogen was studied, binding kinetics and force of individual cells was determined [115]. This technology paved the way of those capable of characterizing and measuring T-cell binding to p-MHC complexes in SPR measurements. Importantly based on these measurements cellular avidity can be determined to various targets [300]. T-cell activation can as well be detected in microfluidic channels by impedance-based assay as demonstrated by Rollo et al. [301] and by dielectrophoresis as well [302]. The application of live cell interferometry to detect antigen-specific cytotoxic T-cells based on quantifying the mass accumulation rate during cell activation was demonstrated. Such techniques enable the identification of single T cell clones recognizing tumor cells in addition to monitoring their cytotoxic effect [303]. Increase in dry cell mass as detected with the same technique can as well be detected for the same purpose [304]. Similarly, quantitative phase microscopy based image acquisition combined with machine learning successfully detected T-cell mediated cytotoxicity [305].

γδ T cells are a unique population of T cells, showing characteristics of T cells and NK cells as well. Seidel et al. showed in a label-free impedance-based kinetic measurement that these cells have both antibody-dependent and independent cytotoxic activity. The method demonstrates the advantages of the kinetic biosensor measurements over the classical end-point assays [306]. Using the same system, T cell mediated cytotoxicity can be monitored as well [307]. NK cell mediated cytotoxicity was studied as well in impedance-based assays. Using real-time electronic sensing system, Zhu et al. demonstrated that in vitro NK cell cytotoxicity can be measured in a kinetic manner using 8 adherent cancer cell line [308]. Fasbender and Watzl carried out fine analysis of this activation using various activating ligands of NK cells on functionalized sensor surface and showed that activation through CD16 was the most prominent and pre-activating NK cells showed and increased response, reaching the peak of CD16 activation using various other ligands as well. Also ligand combinations showed synergistic effect [309].

Park et al. compared flow cytometry and ECIS based cytotoxicity assays and found slightly discrepant results, however they used different target cells [310]. NK cell subpopulations (the mature cytotoxic CD56dim and the regulatory CD56bright) can be distinguished in the microfluidic system designed by Dannhauser et al. based on their light scattering properties with the help of machine learning [311]. Examples of label-free characterization of lymphocytes are shown in Fig. 9.

4.5. Erythrocytes and thrombocytes

Of note erythrocyte and thrombocyte antigens can as well be characterized in immunocapture based measurements on biosensors. High throughput antigen typing is highly desired for transfusion purposes [312]. Coupling the blood type antigen specific antibodies in imaging or classical SPR allows for the accurate and automated typing of red blood cells [313,314]. Although cell sedimentation itself results ambiguous readout, washing with increased flow reveals the specific binding in this approach [315,316]. The successful adaptation of this oligoplex typing approach offers optimism for the future of multiplex, biosensor based clinical developments. Blood group and platelet antigen specific antibodies of auto- and alloimmun origin may result serious pathological conditions [317–319]. Therefore crossmatching to detect alloantibodies especially in those receiving blood transfusion units frequently is a must. Nonetheless, ongoing research to elucidate the mechanism of alloimmunisation and identify those with a higher risk of severe outcome also helped us to better understand antibody effector functions. Ig subclasses, allotypes, glycoforms all differ in their ability to bind Fcγ receptors, as demonstrated by the binding of opsonized red blood cells to sensor bound Fc receptors, and thus may contribute to the pathological process [320,321]. Platelets targeted by allo and autoantibodies increase the risk of thrombocytopenia. In order to identify the contribution of C-reactive protein (CRP) and C1q Kapur et al. immobilized these molecules on the SPR sensor surface and showed that IgG opsonized platelets bind to CRP [322]. To study the affinity of human platelet antigen specific

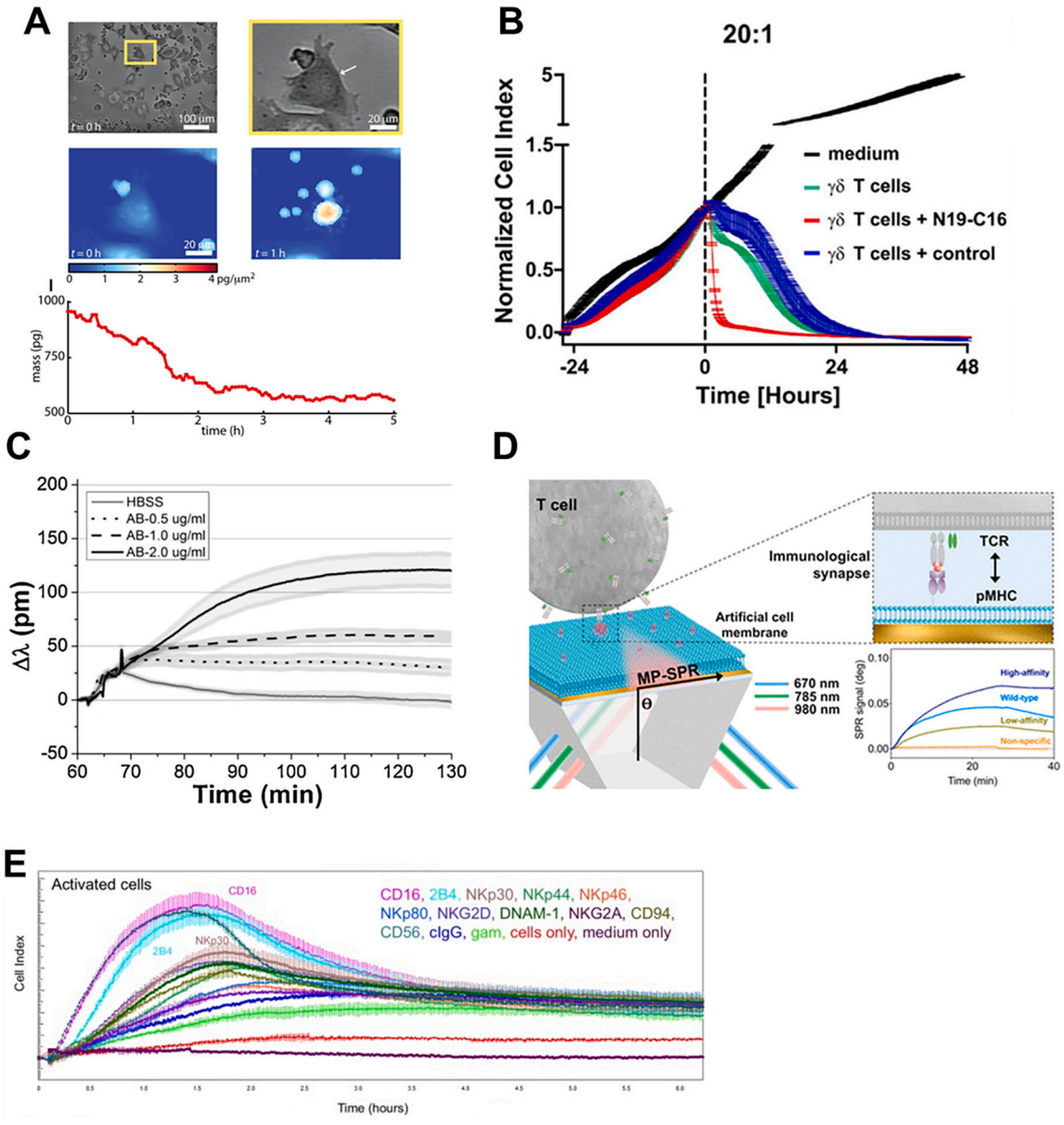

Fig. 9. Examples of label-free characterization of lymphocytes. A, Live cell interferometry based detection of T cell mediated cytotoxicity [303]. B, γδ T cell mediated cytotoxicity of MCF-7-CD19 transfected cells based on impedance measurements in the presence or absence of anti-CD19 antibodies [306]. C, Dose dependent activation of B-cells by BCR cross-linking in RWG based measurements [76]. D, SPR based determination of T-cell pMHC complex interactions reveals TCR affinity [300]. E, Impedance-based characterization of activating properties of various NK-cell ligands [309].

antibodies Wu et al. coupled platelets with amine coupling to the sensor to determine and characterize its correlation with the clinical symptoms and facilitates compatibility testing [323].

5. Conclusions and outlook

These recent efforts demonstrate that biosensor based monitoring of cellular activation on various substrates offers a realistic, low-cost, and most importantly label-free approach to studying leukocyte behavior. Table 1. summarizes the technologies available for biophysical single cell characterization. Novel developments in the field showed that single leukocytes can be studied, characterized and isolated individually. However, it is important to determine the robustness of each assay type and measured parameter and identify those most suitable for diagnostic purposes. Many of the discussed examples provide kinetic data. Deciphering and analyzing these complicated data sets as of now highly demanding on the bioinformatics side, in many case without trivial solutions. In these cases, theoretical modelling of the phenomena is required to fully understand the mechanism of cell activation in real time.

Clinical relevance in diagnostics and cell-based therapy is, however, yet to be demonstrated in most setups. Therefore, most importantly, well-defined samples have to be studied in close collaborations with clinical research groups. Building databases in which clinical data and biophysical variables of cells are combined is the necessary next step in our opinion. Determining healthy range reference of various measured parameters requires large donor cohorts. Moreover, comparative evaluation of the existing methods is desired to characterize cell activation. As even in specialized research centers only a few biosensors are available for a single assay type. Nonetheless, many of the recently developed devices are unique, therefore this step likely requires cooperative initiatives for comparison. Therefore, to fulfill the potential of biosensor based blood cell characterization each cell type is to be studied with the different biophysical techniques and compared to their gold standard methods.

Viability and cell activation status are critical aspects of the sample preparation. Sample preparation by either autonomous or active microfluidics, and microdevices require rigorous testing to minimize cell stress. Varma and Voldman created guidelines on how to protect and assess cell health in microsystems [352]. While technically the sorting of viable cells using microfluidics provides a solution [353–355], and morphological and molecular markers of cell death can be employed to identify dying cells [356], the presence of danger signals may result altered cell activation as well and could possibly interfere with quick, functional testing [357]. Cell viability is also highly affected by the method of cell isolation. We consider minimal preprocessing as the most beneficial. Delay in blood sample processing, transportation or cryopreservation of the samples alter significantly the cell status, and therefore where available whole blood based assays are recommended [358–360].

To achieve this, further improvements are required in blood processing as well, potentially for example chemo- and haptotaxis of the cells could be exploited for the separation of specific subsets [361,362]. Microfluidic systems offer various solutions including topography [363], microfluidic design [364,365] and various membrane based methods as well [366–368]. Furthermore, cells can be separated by size based fractionation [369], by dean flow fractionation or microsieves [370]. The recent review of Laxmi et al. summarizes microfluidic techniques applied to isolate white blood cells [371]. Microfluidic separation and sorting of the cell types of interest is of great interest in various applications [372,373]. Although most of these techniques are microfluidic, the sensing methods can as well be label-free and capable of separation at a single cell level in a nondestructive manner [374–377].

Following impedance-based bulk separation immunocapture allows for further differentiation by cell capture by functionalizing the sensor surface with various antibodies specific for the cell types of interest [378]. Successful SPR based detection of circulating tumor cells using cell surface marker specific antibodies in acute myeloid leukemia has been reported, showing a strong correlation with the flow cytometry-based evaluation [379]. For further details of affinity based cell separation we direct the reader to the review by Zhang et al. [380]. Imaging based approaches, in combination with microfluidics, offer an opportunity to further refine cell sorting. Methods based on Raman scattering microscopy [381], and deformation assisted optical microscopy [382] are two recent techniques that enable high-throughput label-free sorting.

Since cell adhesion, spreading and activation is highly dependent of the assay temperature it is to be tightly controlled. For example in their AFM measurements Rico et al. showed that adhesion force through integrins and tethering decreases with the temperature [383], similarly Sadoun et al. showed increased stiffness and smaller cell adhesion area at room temperature compared to 37 °C [384]. Mechanical properties of monocytes are also affected by the temperature. These considerations emphasize that probing cellular activation, especially POC setups, require temperature controlled biosensing to provide reliable and comparable data.

Several novel techniques measure single cell adhesion force or other parameters and even deposit or select single cells for further examinations (i.e. RNA or DNA sequencing) [332,385].The computer controlled micropipette system is capable of sorting single cells with great accuracy and speed (https://www.cellsorter-scientific.com/). This automated system is mounted onto a normal inverted microscope for probing single cell interactions with specific macromolecules. The adhesion force of surface attached cells can be measured by repeating the pick-up process with increasing vacuum used in the pipette positioned above the examined cell. With this methodology, hundreds of cells adhered to specific macromolecules were tested one by one in a relatively short period of time (~30 min) [58,331]. The application of this technique together with an optical biosensor can be a good combination in single-cell studies [34] or even in experiments with immune cells as well [251]. Not just cells, but also microbeads can be used in adhesion studies. In a recent study, the authors applied the robotic micropipette both in microbead and live cell adhesion experiments to explore the adhesion force of biomolecules (for example cell surface receptors including specific integrins on immune cells) [386].

Selecting special cells is an important technical challenge because individual cells show a high degree of mechanical vulnerability. This problem is overcome by the piezoelectric cell sorting micropipette. This drop-based microfluidic technique is capable of selecting individual cells from almost any sample and prepares them for genetic or other assays. The selection may be based on a fluorescently labeled or unlabeled microscopic image or, a signal from a waveguide sensor. With the fully automated piezoelectric micropipette with a precision of < 1 nanoliter, improving the efficiency of imaging-based single-cell isolation to above 90% [387]. Furthermore, combining biosensor data processing with cell sorting methods allows complex biological and medical experiments to be performed.

FluidFM elevates applications to a higher level, from single-cell biology to surface analysis. FluidFM is basically an AFM device with a nanofluidic channel inserted into the sensitive probe, which is connected to a pressure control system, thus we can control the movement of the liquid column in the channel between −800 and 1000 mBar [388]. Its microfluidic system enables the handling liquid even down to femtoliter volumes (https://www.cytosurge.com/). Soluble molecules can be aspirated or dispensed through a sub-micrometer aperture at the tip of the probe. These developments have opened up possibilities for a wider range of applications, such as the real-time mechanical manipulation of living cellular or bacterial systems, colloidal spectroscopy, and nanolithographic procedures. Latest developments allow even sampling of live cell cytoplasm through the incorporation of a nanofluidic channel in the AFM cantilever, and precise pressure control. Micropatterning of single living cells and cell clusters is also realizable. In a recent work,

Table 1
Summary of biophysical label-free cell analysis methods.

Measurement principle	Measured parameter	Throughput	Image resolution	Time resolution	Sensitivity	Cell type	Information obtained	Manufacturer if commercialy available
Optical waveguide lightmode spectroscopy	refractive index	single sensor	no image	1–3 s	1 ng/cm2	monocytes [250];TERA2 and BHK cells [57]	adhesion kinetics	MicroVacuum Ltd. https://microvacuum.com
Resonant waveguide grating (RWG)	refractive index	96well, 384w	90 μm	3 s	0.078 ng cm^{-2}	primary monocytes, dendritic cells, macrophages [251,324]; B-cell lines [325]; primary B cells [78]; HeLa [65,70]; CHO and A431 cells [64]; HUVEC [74]	adhesion kinetics, signalization kinetics	Corning Inc. https://www.corning.com
Single-cell RWG	refractive index	12 well	25 μm	3 s	0.078 ng cm^{-2}	HT-29 cells [79]; HeLa cells [33,125]	adhesion kinetics, signalization kinetics, adhesion force	Corning Inc. https://www.corning.com
Quantitative phase imaging (holographic microscopies)	refractive index, phase shift	96 well	0.58 μm–4 μm	1–16 frames per second	0.01 RIU 1 pg μm^{-2} Phase detection sensitivity down to 0.011 rad	Hela and Vero [326]; HeLa and MC3T3 [49]; Eosinophil [210];T cell [303]	cell morphology, proliferation, migration, motility, cancer invasion [Nagy in press]	https://phiab.comhttps://telight.euhttps://www.nanolive.ch/https://www.tomocube.com/
Molography	refractive index	single sensor	no image	1 s	200 ng mL^{-1}, ~5 pg mm^{-2}	HEK293 [62]	biomolecular interactions, signalization	Lino Biotech AG https://www.lino-biotech.com
Optical tweezers	adhesion force	1 cell/experiment	microscope image of targeted cells	10^{-4} s	0.1 pN	NK cells [327]; RBC [328]; CD4+ T cells, NK cells, K562 [329]; neutrophils [178,330]	interaction and binding, 3D manipulation, tethered assay	https://www.elliotscientific.com, http://www.impetux.com and home developed setups
Surface plasmon resonance	refractive index	upto 96 well or 400 spots	optical lateral resolution: 25 μm if available	upto 0.1 s	0.15 pg mm^{-2} or $1–10*10^{-7}$ RIU	monocyte [252], RBC [313,314,316], basophil [213,217]	adhesion kinetics, signalization kinetics	https://www.cytivalifesciences.comhttps://www.bionavis.comhttps://www.ibis-spr.nl https://www.horiba.com/http://www.unilim.fr/pages_perso/zeng/a17.pdf
Electric cell-substrate impedance sensor	impedance	96 and 384 well plates	no image	10^{-4} s	under 10 to 10,000 Ω cm2??	NK cells [309], γδ T cells [306]	proliferation, migration, attachment and spreading, differentiation, cytotoxicity, inflammation, invasion, barrier function	https://www.biophysics.com https://www.agilent.com/en/product/cell-analysis/real-time-cell-analysis
Computer controlled micropipette	pressure difference, adhesion force	100–200 cells/h	microscope image of targeted cells	10–30s/cell	nN range	macrophages, dendritic cells, monocytes [331]; monocytes and 3T3 [332]; primary monocytes, dendritic cells, and macrophages [251,324]	adhesion strength	Cellsorter https://www.cellsorter-scientific.com/
FluidFM	force	upto 200 cell/day	microscope image of targeted cells	minutes/cell	nN range	HeLa [33,333,334]	adhesion force, *Note, the technique is suitable for cell injection and extraction [335,336]	Cytosurge https://www.cytosurge.com/
Raman based technologies	infrared spectra	couple of cells	0.3 μm, * Note, the lateral resolution can be significantly enhanced by tip	~1 s per sampled point	nN range	macrophages [262]; neutrophils [176]; T and B cells [338];T and B	cell identification, characterization of cell status through determination of	https://www.enwaveopt.com/https://bwtek.com/ https://www.horiba.com/int/scientific/pr

(*continued on next page*)

Table 1 (*continued*)

Measurement principle	Measured parameter	Throughput	Image resolution	Time resolution	Sensitivity	Cell type	Information obtained	Manufacturer if commercialy available
			enhanced raman spectroscopy [337]			cells, monocytes [339,340]	biochemical composition	oducts/raman-imaging-and-spectrometers/labram-soleiltm-raman-microscope/
Surface acoustic waves and acoustic force spectroscopy	force	thousands of cells per measurement	microscope image of targeted cells	s-min	1 pN	T cells [115], CAR T cells [341,342]	cell avidity	https://lumicks.com/
quartz crystal microbalance	resonance frequency	4 channel and 8 sensors (QSense Pro QCM-D)	no image	1–3 s, Biolin: up to 200 data points per second	0.2 ng/cm [2]–1 ng/cm [2] - ~0.5 ng cm − 2	RBC [343]; macrophages [253,254]	biochemical reactions, cell adhesion and detachment, deformability	MicroVacuum Ltd. https://microvacuum.com/ Biolin Scientific AB https://www.biolinscientific.com Attana https://www.attana.com
traction force microscopy	force	1–5 in single cell experiments, several hundreds of cells in collective migration studies	microscope image of targeted cells	10^{-4} s	no data	macrophages [344,345]	migration, adhesion force, mapping the traction field of cells	custom made platform, can be adapted to commercial optical microscopes
Atomic force microscopy	deflection of the cantilever, force	10 min/ whole cell	topographic image with nm resolution depending on fixation	topography is faster, elasticity measurement and evaluation is time consuming	1 pN	T cell [346];neutrophils and macrophages [347]; THP-1 and CHO cells [348]	topography, elasticity, mechanical properties	https://www.nanosurf.com/en/ https://www.hitachi-hightech.com/global/ https://www.bruker.com/en.html
dielectrophoresis	dielectrophoretic activity	upto 160,000 cells/s	no image	not defined	not defined	RBC, fibroblast [349]; HeLa, Jurkat, MCF-7 [99]	cell sorting	http://www.siliconbiosystems.com/technology-products
Impedance flow cytometry	electric properties	upto 1000 cell/s	no image	not defined	not defined	all blood cell types, WBC RBC separation [350]	cell sorting	https://amphasys.com/impedance-flow-cytometry/#ifc
Grating-coupled interferometry	refractive index	4 channel	no image	150 ms transition time	0.015 pg/mm2	yet only demonstrated for bacterial cells [351]	ligand-analyte integration and kinetics	Creoptix AG, Malvern Panalytical https://www.malvernpanalytical.com

micropatterning of living mammalian cells (HeLa) on carboxymethyl dextran hydrogel layers using the FluidFM BOT technology [389]. The combination of this technique with optical RWG biosensor to monitor cancer cell adhesion significantly increased the measurement throughput, and opened the way to combine the technology with the employed microplate-based, large area biosensor [33].

Studying population level heterogeneity at the single-cell level using these novel biophysical technologies, and identifying important sub-populations will potentially open up new research directions with important aspects in medical diagnostics, and further treatment. Single-cell optical biosensors [33,390] and high-throughput robotic FluidFM [333] were already successfully demonstrated in monitoring population distributions of single-cell biological parameters in a label-free and noninvasive manner. Importantly, concerning adhesion strength, a lognormal distribution profile was revealed [33,333,390]. Therefore, treating the cell population as normally distributed or measuring only a few cells can easily result in misleading conclusions.

In a recent study, FluidFM was combined with computer controlled micropipette as well to measure the adhesion strength of microbeads. Furthermore, the bead-support contact zone was directly characterized on an optical waveguide biosensor to determine the density of avidin molecules. The authors found that both methods provide unimodal histograms. FluidFM BOT can directly measure the detachment force curve of 50 microbeads in 150 min, and automated micropipette can provide calibrated binding/adhesion force values of 120 microbeads in an hour [391]. These methodologies could be extended to measure leukocyte surface interactions with pre-coated microbeads with biological relevancies. The proposed approach would enable the single-cell level characterization of the interaction forces with unprecedented throughput, and depending on the size of the microbeads, might offer some single cell level lateral mapping of the interaction forces, too. Unlike in other setups, the beads could be easily exchanged, mapping in this way the interactions with more than one biological coating on exactly the same cells.

The logical follow up of the measured cells is to perform molecular analysis. Matching biophysical parameters at a single-cell level with metabolomic, proteomic [392,393], genomic and transcriptomic [394,395] information provides verification and allows for comparison of the methods [396,397].

Combined with microfluidic platforms designed to process whole blood, the era of point-of-care diagnostics is certainly around the corner. It will be interesting to see whether these applications can be combined in the future with microneedle based approaches to continuously monitor various biomarkers of interest [398]. Implementation of smartphone based biosensors is highly desired [399], and as discussed, microfluidic sample preparation combined with label-free detection could as well be applied for blood cell based diagnostics in self-contained microfluidic systems [400].

The application of label-free techniques is expected to be more pronounced in the future. Compared to traditional methods based on structural or/and composition analysis, which are usually endpoint assays, the real-time kinetic measurements without introducing any disturbing agents offer an interesting novel way of obtaining biological information. The specificity of the measurements might be weaker compared to traditional methods, but the rich data sets generated, especially in combination with artificial intelligence based decision making, can offer new routes in both basic researches and in the health industries. We also predict the emergence of more complex setups where the label-free sensing and the high-throughput single-cell isolation are effectively combined. These combinations not only can offer a simple and cost-effective cell-identification route, but allow for the isolated live cells to be readily used in further processing or culturing steps.

Declaration of Competing Interest

The authors declare no conflict of interest.

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