Chapter

Real-Time Tracking of Individual Droplets in Multiphase Microfluidics

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Abstract

Multiphase microfluidics enables the high-throughput manipulation of droplets for multitude of applications, from the confined fabrication of nano- and micro-objects to the parallelization of chemical reactions of biomedical or biological interest. While the standard methods to follow droplets on a chip are represented by a visual observation through either optical or fluorescence microscopy, the conjunction of microfluidic platforms with miniaturized transduction mechanisms opens new ways towards the real-time and individual tracking of each independent reactor. Here we provide an overview of the most recent droplet sensing techniques, with a special focus on those based on electrical signals for an optics-less analysis.

Keywords: droplet microfluidics, lab-on-a-chip, nanosensors, biosensors, field-effect transistors

1. Introduction

Implementing new tools for bioanalytics is the priority goal for the modern diagnostics and medicine. This task can be efficiently fulfilled only by merging the expertise from various research fields, that is materials and nanoscience, bio- and electrical engineering, chemistry. In this respect, a microfluidic concept that groups the tools and techniques to study and manipulate fluids at a submillimeter length scale, represents an ideal solution for micro- and nanosensors-based platforms. The field of microfluidics has shown the power of downscaling techniques for the manipulation and analysis of fluids [1]. High-throughput characterization of processes in such applications is a key task, particularly in medical diagnostics, food processing and pharmaceutical industries [2]. Although downscaling is in principle compatible with parallelization to increase the throughput, implementing a large number of experiments would require a dramatic scaling-up of the overall platform size. The solution to this issue is found in the droplet microfluidics technology. Here, immiscible phases are used to create discrete volumes of confined solutions, maintaining the small footprint of the employed device [3].

The generated emulsions can be counted even up to thousands per second [4]. With each single droplet being an independent miniaturized laboratory for biochemical experiments, this technology allows highly parallelized and controlled measurements, surpassing the precision of conventional assays. Other advantages benefit the droplet generation systems, such as enhanced mixing and mass transfer efficiency leading to faster reaction times [5] or minimized sample absorption on channel walls [6].

Since the first demonstration in 2001 [7], this novel bioanalytic approach has found a wide range of applications: generation of drug-loaded particles for therapeutic agent delivery [8], fabrication of micromotors [9], confinement of particles [10] creation of synthetic cells [11], microbubbles as ultrasound or photoacoustic contrast agents [12], diagnostics [12], microcapsules for cell culture [12], etc. In particular, the approach has proved itself effective in microbial cell assays for both fundamental microbiology research and clinical studies, *i.e.* to determine the metabolic activity, division rate, and drug resistance [13–15]. Although important contribution have been made, the technology remains a work in progress. Strong efforts are still invested in the development of droplet tracking and analytical techniques. Real-time detection and analysis of the droplets is critical to monitor the dynamics of the ongoing confined events. Additionally, real-time analysis enables a timely response to the needs by tuning the properties or the direction of the generated droplets as needed. A simple photography or video acquisition process could provide access to basic features such as droplet size, morphology, color, or rough monitoring of microscopic objects inside. Here, the processing power may impose a limitation to the amount of droplets per second that can be analyzed. Implementing alternative transduction mechanisms could offer a much simpler setup with faster sampling rate. It could also give access to information on the nature of the droplet content, which cannot be easily obtained from an image.

Here, we provide an overview of the existing approaches for the real-time tracking of droplets in multiphase microfluidic devices. We make a distinction between optical and optics-less techniques, with electrical or electrochemical and magnetic techniques as part of the second group, which can find droplet properties that remain more hidden to the eye. While there are additional techniques already demonstrated such as mass spectrometry [16] or electrospray ionization mass spectrometry [17], we put the focus on the former two groups of techniques as more advantageous ones in terms of miniaturization possibilities in the spirit of the lab-on-a-chip purposes.

2. Optical methods

The analysis of optical signals, *i.e.* photoluminescence and optical density (OD), is currently the conventional state-of-the-art method to detect and monitor the droplets in a microfluidic format. A schematic representation of a microfluidic platform equipped with an optical detection system and its operation is given in the following **Figure 1**. The complete system is capable to perform multiple tasks, which are divided into the separate modules, *e.g.* formation of the droplets, their transportation, incubation, merging, splitting, etc. This enables to mix the reagents in droplets and to tailor their composition on demand. Exemplary, the droplets are generated using *e.g.* flow focusing geometry, via controlled emulsification of the aqueous phase using oil containing surfactants for stabilization. The droplets are typically generated at few μm-scaled flow-focusing junction (*ca.* 10–30 μm) and are transported in a few mm long channel before droplets reach further manipulation structures [20]. Manipulation of the droplets content, size and interfacial properties are usually used for example to initiate multitude of chemical

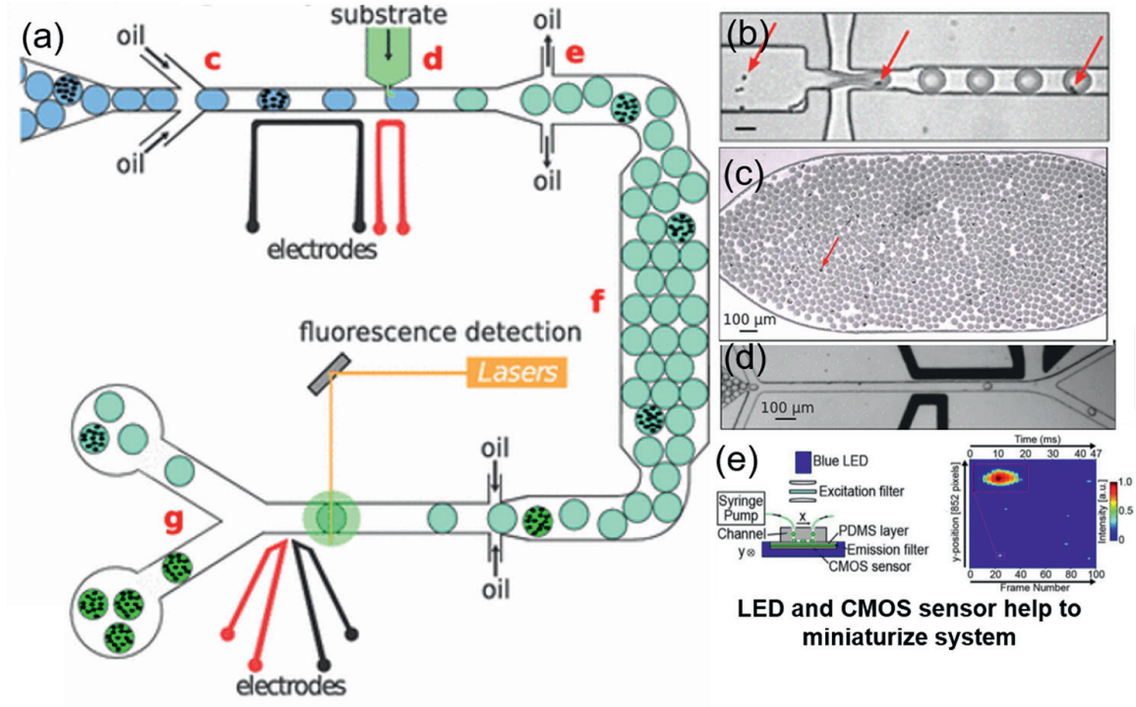

Figure 1.
Complete system for (a) droplet processing, (b) generation, (c) storage, (d) sorting, and (e) image analysis. Adapted with permission from refs. [18, 19].

reactions [20] of interest. This is performed via fusion of the droplets that is controllably afforded using electro-coalescence [21]. For the multiparametric on-chip analysis, droplets may be split into the fractions at the multibranch junctions. The light microscope images show the respective processes of the droplets generation, merging, splitting, etc.

Efficient detection of thousands of droplets one-by-one and providing of the sufficiently high signal to noise ratio (SNR) is of extreme importance in droplets based systems. **Figure 1a** shows the schematic representation of the typical light path configuration across the microfluidic channel, applicable to detect droplets in microfluidic systems. For these purposes, the optical microscopy with the integrated illumination, *e.g.* a mercury arc or halogen lamp, integrated semiconductor lasers [22, 23], laser emitting diodes (LEDs) [24], are used as the light sources. A semiconductor laser is considered to be one of the most frequently used tool, being able to produce monochromatic, directed light beam, staying relatively compact. To enable the fluorescent analysis, setups are supplemented by the combinations of the filters and lenses for focusing and filtering excitation and emission beams. Apart from the light source, typical detection event involving fluorescence, *e.g.* to count droplets, employs pair of the dichroic mirrors (DM) to assure the precise excitation and emission of the light. Reagents in droplets are excited on the fly, during exposition to the light beam for *ca.* several milliseconds [25]. The intensity of the emission is proportional to the intensity of the incident light. Emitted light is directed by the respective optic elements, such as fibers and mirrors, towards detectors, which are represented by photomultiplier tubes (PMT) or avalanche diodes. The PMTs and diodes convert the light into an electrical signal for the detection of fluorescent events one-by-one. This configuration typically results in a high-throughput benchtop instrument. Although lasers provide great SNR for droplets detection, they miss the cost efficiency, low energy consumption and miniaturized integration, which is a specific peculiarity, for example of LEDs. It should be however noticed that for the sake of setup simplification (*e.g.* skipping

the excitation filters), only narrow-band LEDs are suitable. Overall, thanks to the aforementioned advantages LEDs are currently the most used technology for dynamic detection of the droplets in micro- and millifluific setups. Fluorescent microscopy with the integrated Charge Coupled Device (CCD) sensors and CMOS sensors are still actively employed to offer spatial monitoring of the droplets-fluorescence over time [18] (**Figure 1e**). This has high relevance for the realization of the droplet-based digital Polymerase Chain Reactions (PCR) and next generation sequencing for monitoring of *e.g.* circulating tumor DNA [26]. In this realization microscopy scanning of the multiple droplets that are stored in the incubation chamber is performed in time domain. Droplets with the positive amplification of the fluorescent signal are visually detected, as demonstrated in **Figure 2** [27].

2.1 3D detection of droplets

Aforementioned systems assist in the highly sensitive detection of droplets, quantifying the signal that reflects the distribution of the fluorescence intensity in droplets within a focal plane of the optical lens. In order to get the spatio-temporal distribution of the fluorescent signal 3D image acquisition is required [28]. Therefore, a novel merge of droplet microfluidics with the light-sheet fluorescent microscopy (LSFM) was proposed, with the aim to achieve high-throughput compartmentalization, manipulation and three-dimensional imaging of the sample. This was realized via integration of the orthogonal plane illumination into the optofluidic system. Each droplet that moves through the detection area of the device was scanned by a laser-sheet, three-dimensionally reconstructed and analyzed. It consisted of an upright droplet microfluidic chip and a horizontal laser-sheet illumination path, where the laser-sheet penetrated the micro-channel transversely. As one droplet flowed down through the detection region, it was optically detected in sections by the thin laser-sheet in a way of automatic fluid scan. The fluorescent signals from the sequentially illuminated planes of the droplet were then measured by the microscope's objective from the side facet of the chip and recorded using a high sensitivity CMOS camera. The optical microscope with a large field of view objective recorded the fluorescent signals with a high acquisition rate of 500 fps, and z step-size of 3.5 μm were reached.

To track fluorescence within every droplet, the solution of colloidal particles doped by a fluorescent dye was used during encapsulation. The 3D detection of such droplets, formed with the smaller and smaller sizes (from 1.5 mm to 0.5 mm), is demonstrated in. Each droplet consists of over 500 3D stacked plane images from different depths of the encapsulated particle clusters. Generally, this technology opens great potential for various lab-on-a-chip studies, such as embryo sorting and organoids growth monitoring, etc.

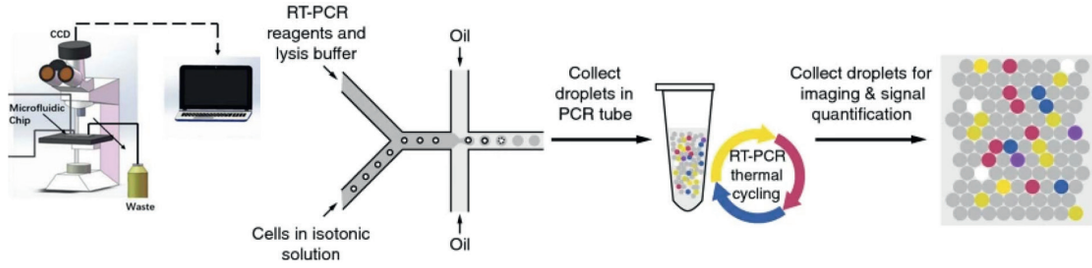

Figure 2.
PCR microfluidics with microscopic analysis of fluorescence development. Adapted with permission from ref. [27].

2.2 From micro- to millifluidics

The use of femto-picoliter droplets is crucial in a number of experiments, such as biomolecular assays or molecular evolution. However, the upscale of the technology is necessary, when investigating the cells metabolism or their response to one or several stress factors. In this case, the behavior of populations over several generations of cells has to be monitored. This format of the experiments is possible, when the aqueous reactors (droplets) contain sufficient amount of *e.g.* nutrients and other supporting species. In contrast to more common microfluidic approaches, millifluidics appears as an approach for quick screening of larger volume aqueous samples for any quantitative analysis of its biological and chemical content. For comparison: while microfluidics work with picolitre reservoirs, droplets in millifluidic systems reach volumes in the range of 50–200 nL [29]. Therefore, the technology was adapted respectively, by switching from microchannels used in polydimethylsiloxane (PDMS) chips to commercially available off-shelve components, *e.g.* transparent fluorinated ethylene propylene (FEP) tubes with an inner diameter (ID) of about 0.5 mm. Thus, millifluidics emerge as a tool, crucial for a long term monitoring of a large number (up to 10^3) of biochemical reactors containing cell or bacterial populations. The millifluidic technique [13] in this particular case would outperform the microfluidic approach in terms of its better suitability.

In the following we demonstrate several examples of the millifluidic systems that are used to incubate the microbes, investigate their antibiotic susceptibility and even to study their coexistence processes. In these realizations authors demonstrate the fluorescent detection principle, based on the multiple readout of the droplets one-by-one to build up the time dependent curves of the process kinetics [29–31].

Figure 3 demonstrates the typical optical and fluidic setup assembly that is used to fulfill these goals as well as its capabilities [30]. Millifluidic setups typically offer the automatic droplets readout to perform large-scale high resolution assays and calibration. Panels a–d in **Figure 3** depict a schematic diagram of the system, which is divided into two main areas: droplet generation and detection. Sets of optomechanical elements and polymeric capillary tubings are used to assemble such system, being controlled by the custom LabView software. This program enables controlled droplets generation, counting, detection, back-forth motion for repeatable scan of every droplet in the chain. In the detection area, a double fluorescent detector is designed to be responsive to the blue and yellow fluorescent proteins (BFP and YFP), simultaneously (see panels d–g in **Figure 3**). A fluidic pump controls the droplet sequence flow forward and backward to the detectors by infusing and refilling the fluidic circuit. LabView software can automatically measure the growth curves of two strains with high precision during hours and days (**Figure 3e–g**). In the following, we applied the setup for the quantitative recording of the growth curves from the multiple droplets, including the co-culture of two strains of *Escherichia coli*, revealing fluorescence according to the BFP and YFP emission spectra. After determining the relationship between fluorescent signal and cell density for each strain, calculation of the limits of detection (LODs) of the droplets analyzer was performed, with LOD for *E. coli* BFP around *ca.* 5000 cells per droplet, and for *E. coli* YFP around 6000 cells per droplet. Growth kinetics of bacteria both, in monoculture (**Figure 3e–i**) and co-culture (**Figure 4**) were accurately measured, with various initial cell densities (inoculum). Interestingly, the setup opens possibility to study the organisms' cooperation and coexistence at small scale, by varying the fraction of each bacterial strain inside of the droplet, that affects the final growth curves for these strains. Furthermore, such system represents the elegant way to form a gradient

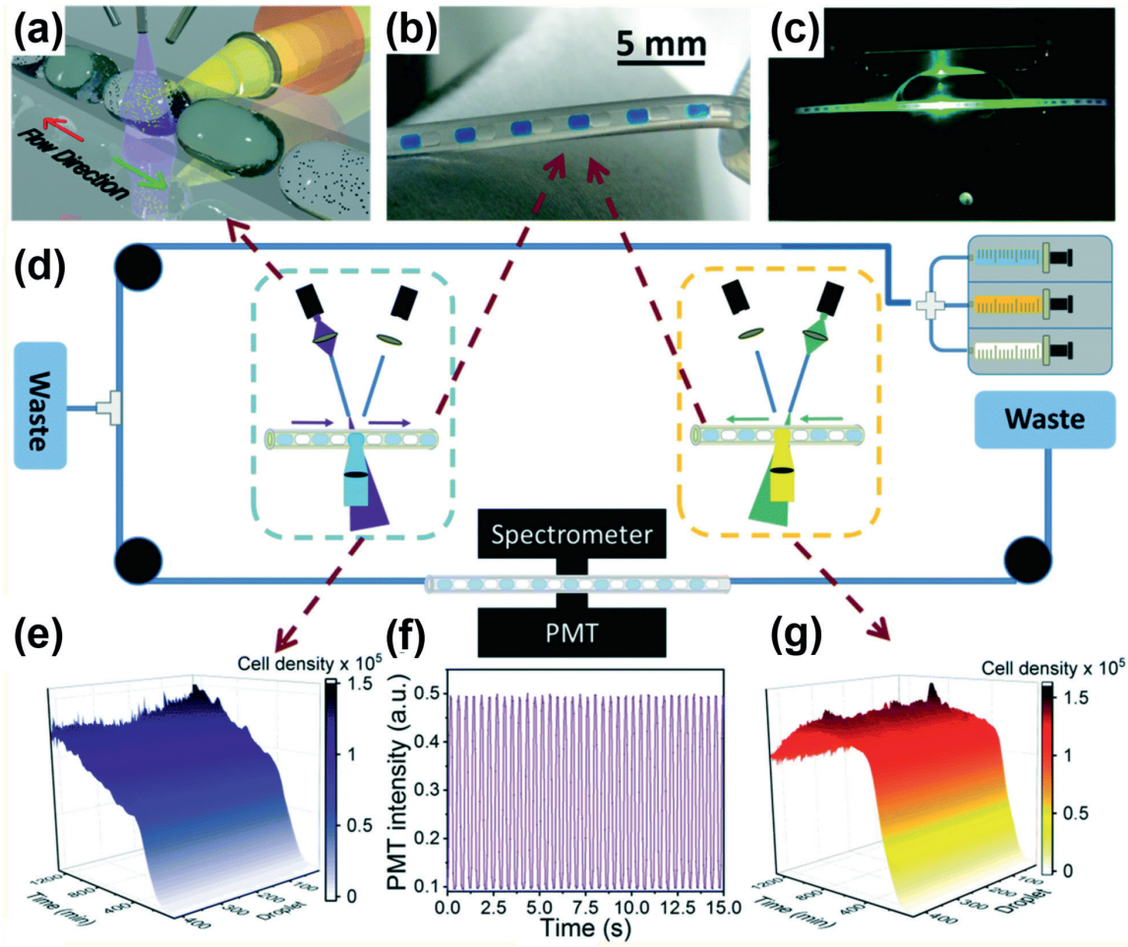

Figure 3.
Millifluidic setup for fluorescent bacteria detection. (a) Schematic of the detection mechanism. (b) Photography of the generated droplets and (c) their fluorescence measurement. (d) Schematics of the double detector for the analysis of two wavelengths, each belonging to a different cell strain: E. coli YFP and E. coli BFP. (e) Growth curve for E. coli YFP monoculture, with example droplet peaks in (f). (g) Growth curve for E. coli BFP monoculture. Adapted from ref. [30].

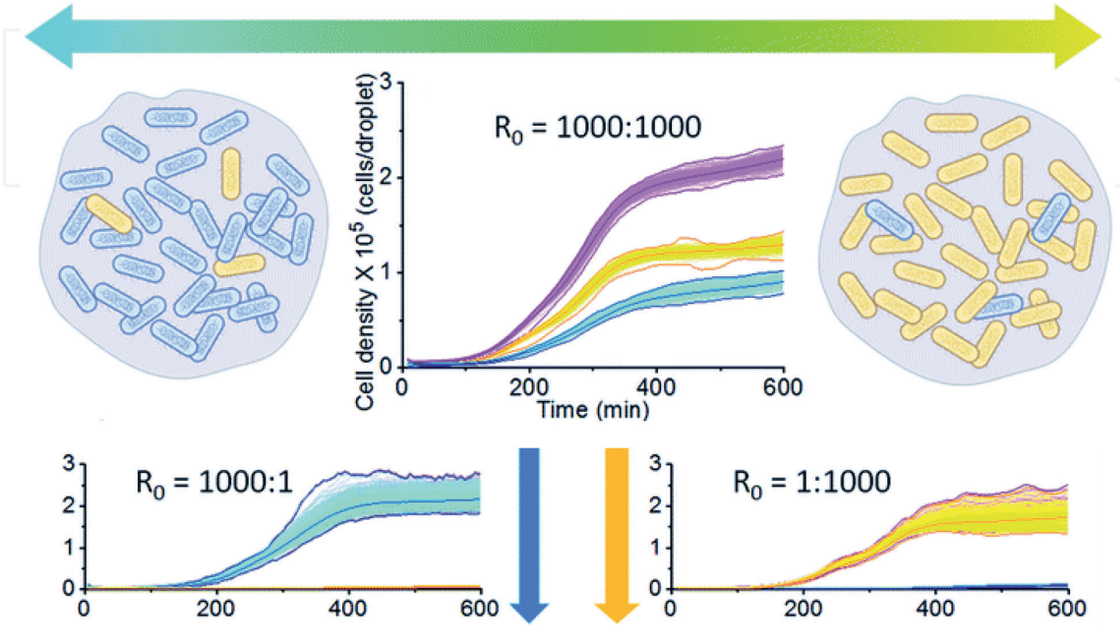

Figure 4.
Growth kinetics of cocultures from different initial cell density ratios. Adapted from ref. [30].

of the chemicals, *e.g.* nutrients, drugs, etc. along the droplets chain and challenge the microbes to survive in this stressful environment [29]. This opens the door towards measurements of bacterial resistance to *e.g.* antibiotics, which are of interest for microbiological and clinical applications.

2.3 Outlook

Overall, optical tools played very important role in the establishing and development of the droplets based microfluidics, and the lab-on-a-chip field overall. However, although being very efficient optical approaches still stay bulky. Therefore, one of the main challenges today is the need for the development and integration of novel miniaturized optics-less detection principles [32, 33], outperforming or being complementary to the conventional approaches. In contrast, new devices make the measurement processes independent of the limitations of optical microscopy, *i.e.* dynamic range or use of molecular labels.

3. Electrical and electrochemical methods

Detection and analysis of droplets using electrical or electrochemical transduction mechanisms have the potential to reach speeds difficult to achieve by conventional optical means. Furthermore, implementing miniaturized transducers that can be applied in low-cost portable devices such as the lab-on-a-chip approach can be more easily implemented by getting rid of the optical elements. The nature of the detection is label-free, as compared to certain optical techniques like fluorescence, which also suffers from photobleaching. Performing multiple parallel measurements by using various sensors in a single chip is also enabled more easily. **Figure 5** shows examples on their positioning, either as sensors at the bottom of the channel or parallel plates to surround the droplets, or some examples of the types of transducers that can be used, from simple planar or parallel electrodes to field-effect transistors (FETs), resistors, etc.

As shown by early demonstrations, access to basic information such as presence, size and ionic concentration [34] is possible through the direct contact of conductive droplets with coplanar electrodes at the bottom surface of the microfluidic channel, providing a signal in opposition to the insulating nature of the carrier phase, either air

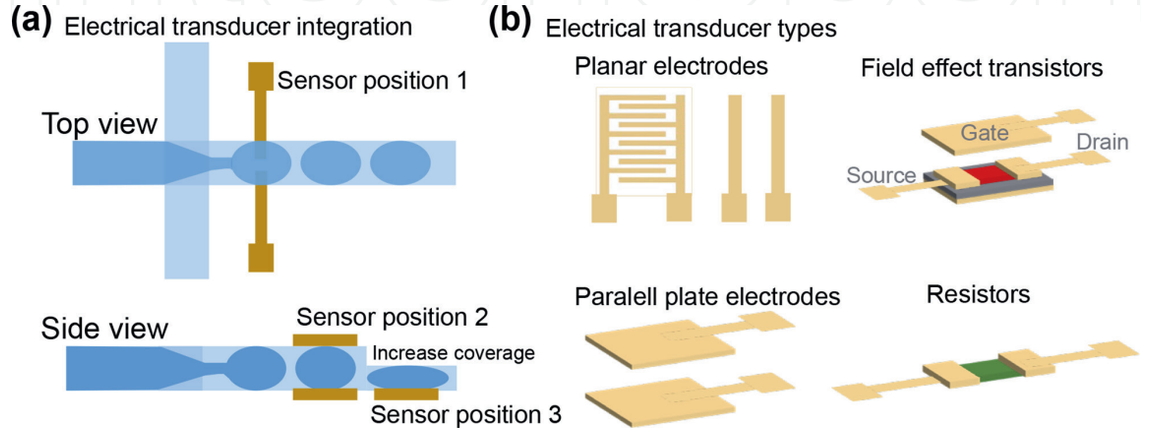

Figure 5.
Electrical transducers in droplet microfluidics: (a) examples of their integration in the bottom of the channel or as parallel plates, including narrowing the channel for increased sensor coverage, and (b) examples of transducer types.

[35] or oil [36]. Size and velocity can be deduced measuring the time taken to travel between or through electrodes and the dimensions and distance of these electrodes. Differences in thermal conductivity between the two phases can also provide the information at the cost of a more difficult fabrication approach requiring resistive serpentine resistors [34]. Although some of such early works could implement the droplet sensing systems in microdevices where complex biological experiments were carried out (*e.g.* yeast cell electroporation [36] or DNA amplification through PCR [37]), the analysis of the biological and biochemical events occurring inside the droplets was still done via traditional microscopy or cameras. The sensors could for example be used to indicate the location of the droplets, and an automated position control generated pneumatic pulses to prevent the escape of the droplets from the reaction area. Such work reported that biological material can be lost through such direct contact with the sensor area, which could cause cross-contamination as well as decrease in the yield of the reactions.

The passing of droplets nearby electrical sensors resembles to some extent the impedance flow cytometry, where cells change the resistive and capacitive components of the signal [38]. Inspired by such technology, variations in the different components of the impedance can be used as source of information for droplet analysis. Another early demonstration [39] reported a capacitive detection system that could analyze droplet composition depending on its dielectric constant, in addition to droplet size and speed. Here, the detection system was coupled to a feedback loop for droplet sorting up to 10 kHz. This detection rate was not limited by the measurement speed, but by the upper pressure limit to fabricate the droplets without leading to leakage at the inlets ports. The capacitance of parallel electrodes on the side walls of microfluidic channels changed with the passing of the droplets due to the difference in the dielectric constant between droplets and carrier liquid. Such difference is a requirement for this type of sensing, which is the case for droplet microfluidic applications consisting of water-in-oil emulsions. In order to fabricate sidewall electrodes, a multistage photolithographic process was necessary where one of the steps involved filling photoresist cavities with a PDMS gel containing silver micro/nanoparticles to form the electrodes. The final microfluidic chip was integrated in a custom made circuit board containing an ac waveform generator, an L-C resonance circuit, an amplifier, an ac-dc converter and a comparator to generate the feedback control for the droplet sorting. When droplets have a correctly developed symmetric geometry, the sizing and speed calculation is straightforward with one simple pair of electrodes by evaluating the time taken to reach a plateau in the signal and the duration of the plateau. Since the difference between long and short droplets is mostly the signal amplitude, the size and speed can also be measured by evaluating the time taken to pass from one pair of electrodes to the next pair. Similar results can be obtained if a single electrode pair with fork shape is used (**Figure 6a.i**). Here, the plateau shows a central peak for large droplets and a dip for the small ones, helping to determine size and velocity. Since the principle of the technique is the difference in dielectric constant between the carrier fluid and the droplets, the chemical composition will also lead to signal variations, as shown by the authors using water and ethylene glycol droplets as proof-of-principle (**Figure 6a.ii**). In their device, the authors used a first pair of electrodes to determine velocity, size and content, while further pairs with different polarization (determined by the output signal of the first pair) attracted or repelled droplets to the different branches in the microfluidic channel layout. The required minimum effective electric field was given by the hydrodynamic flow resistance, while for too high electric fields the water droplets were stretched into satellite droplets.

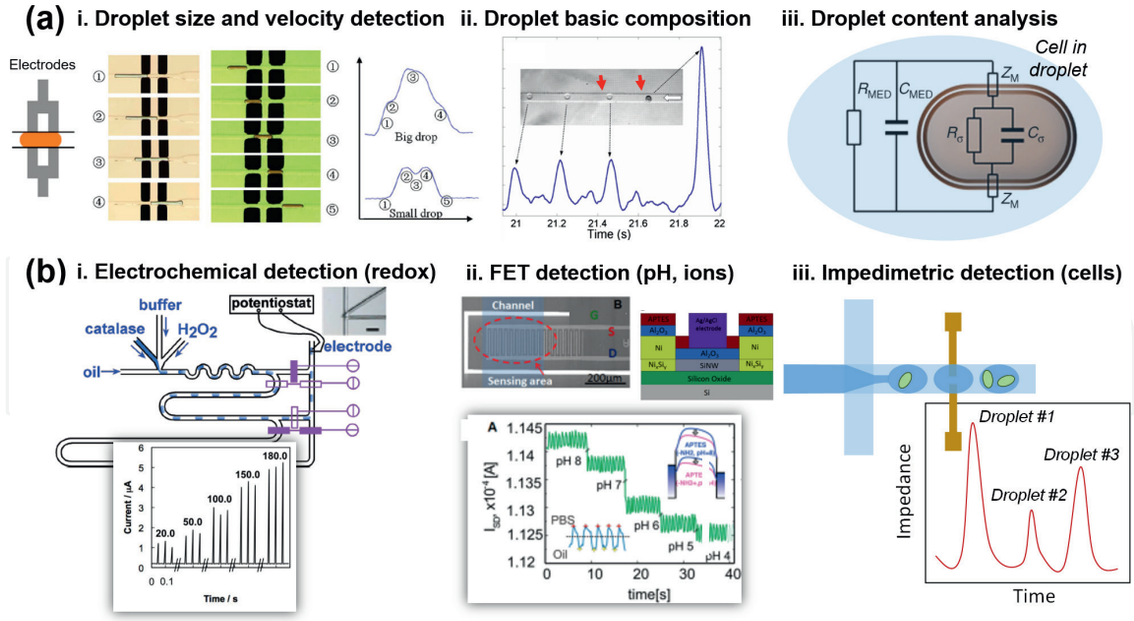

Figure 6.
Application examples of electrical droplet sensing: (a) type of information to target: i. size and velocity, ii. Composition of the liquid forming the droplet, iii. Presence of entities in the droplet (cells, particles, etc.). (b) Type of content to measure depending on transduction mechanism: i. redox reactions with electrochemical sensors, ii. pH or ions with FETs, iii. Cells with impedance sensors. (Adapted with permission from ref. Niu et al. [39]; Kemna et al. [40]; Schutt et al. [41]; Han et al. [42] and Spencer et al. [43].)

Although parallel electrodes are expected to have better sensitivity, capacitive sensors based on coplanar electrodes can perform the task as seen before. Their fabrication is simpler, faster, and they are already commercially available. A previously reported work demonstrated the implementation of commercial interdigitated electrodes with an analytical model to consider the effect of the passivation layer that prevents the cross-contamination issue through the direct contact between droplet and electrodes [44].

By a more complex analysis of the signal which includes not only capacitance, but the whole impedance signal including the resistive component as well, deeper insights on the droplet interior can be obtained beyond size, velocity and composition of the liquid forming the droplet. If the electrical detection of droplets functions similarly to impedance flow cytometry for cell detection and analysis, the presence of cells in droplets should also be feasible (**Figure 6a.iii** and **b.iii**). This application was demonstrated for the first time using planar electrode pairs functioning at 100 kHz for high speed detection. The authors used the technique to discriminate droplets containing viable mouse myeloma cells from non-viable ones. The 100% yield of viable cell-containing droplet generation is not possible if a fast detection technique is not used to make the sorting possible. They showed that empty droplets with low conductive medium could not be distinguished from those containing non-viable cells, due to the resistive effect of their membrane. On the contrary, droplets containing viable cells resulted in lower impedance signals due to the presence of the conductive cytoplasm. As aforementioned for the capacitance sensors, when a droplet appears the capacitance values change. In addition, the resistance is also different for the oil and buffer. Additional equivalent circuit elements appear with the presence of cells, which include the capacitance of the membrane and resistance of the cell interior. Under the megahertz frequencies, the membrane of the cells usually imposes a barrier to the current flow and the change in the impedance signal will be dependent on cell size [45]. However, if a low conductivity buffer is used, the current can flow through the cell at lower frequencies [46].

Considering that at low frequencies the double layer capacitance dominate, the measurements should then be made at intermediate frequencies where the resistance due to liquid composition (including cytoplasm) play a role. The authors could demonstrate as well that the electric field did not negatively affect cell viability.

Certain biological and chemical processes (*e.g.* enzymatic reactions) present rapid kinetics in the milliseconds range and with especial prominence in their very first moments. To achieve maximum detail in the kinetics, fast detection techniques are a must here. In this context, chronoamperometry can be a faster alternative compared to impedance, requiring as well a simpler operation and readout setup [47]. The signal in amperometric sensors is generated by the exchange of electrons between the electrode and the analyte or the receptor at the electrode surface interacting with the analyte. The analyte is involved in a redox reaction, changing its oxidation state and producing a measurable electron flux, proportional to the amount of species transformed [48]. The targeted type of samples is therefore limited to electroactive species. An additional limitation comes from the contact time between electrodes and droplets. When this time is too short, the faradaic current originated from the redox reaction is convoluted with a large capacitive current [49]. To ensure enough contact time, large droplets or fluid plugs can be generated (therefore decreasing the throughput), or the microchannel section can be narrowed down at the area where the electrodes are located, stretching the droplets and improving as well the intra-droplet mass transfer characteristics [50]. Another solution was found by enhancing the wettability of the detection area to convert the droplet flow into a vertical laminar flow, where the oil phase flows on top of a continuous aqueous stream only during the measurement [51]. The chronoamperometric technique has been used for example for the measurement of the Michaelis–Menten kinetics of the hydrogen peroxide decomposition by the enzyme catalase [42]. Here, the route of the droplets was controlled by pneumatic valves, from short to long, allowing to measure at different time intervals of the reaction. The H_2O_2 was oxidized at the surface of platinum electrodes polarized at 600 mV, decomposing into protons, electrons and oxygen. The diffusion was not a limiting factor, as observed by an independence of the current peak from the flow rate. The current amplitude depended from the concentration of the analyte. A time resolution of 0.05 s was achieved, consuming less than 50 μL (**Figure 6b.i**). The amperometry technique combined with microfluidics allows to get deeper insights into enzyme analysis such as inhibition assays, which would otherwise require time consuming serial dilution experiments involving 1000-fold larger reagent volume. The microfluidic channel can be modified to perform additional tasks such as the generation of concentration gradients in order to analyze the dose–response assay with enzymes and inhibitors at different concentrations, as shown with acetylcholinesterase and various drugs (pesticides and therapeutic drugs for Alzheimer's disease) [52]. Here, the measurement principle consisted on the oxidation of thiocholine as product of the enzymatic activity. The authors suggested that the presence of a surfactant as droplet stabilizer can be an important factor to consider. Surfactants can cause protein adsorption at the interface between oil and droplet, decreasing the efficiency of the reactions. The droplets can remain stable for several minutes, which can be enough for enzymatic activity assays, but the presence of a surfactant is necessary in longer incubation periods, needing investigations in order to choose the most appropriate one. The authors could determine the half maximal inhibitory concentration (IC_{50}) in the μM concentration range during a final assay time of only 6 minutes. When nanomaterials are part of the electrode, the signal has been observed to enhance at least an order of magnitude [53, 54].

Potentiometry can also be considered as a fast technique offering time resolutions comparable to those obtained by amperometry. Certain works report the use of light-addressable potentiometric sensors, where a light source excites and generates carriers on a semiconductor device, producing a potential that will be modified by the presence of charged species at the surface [55, 56]. However, in this section we will focus on the optics-less systems which get rid of the optical elements for an easier miniaturization. Potentiometric sensors that have been used for droplet detection can be mainly categorized into the next two types: ion-selective electrodes and ion-sensitive field-effect transistors (FETs). The fabrication process and the setup used in the first type can be as simple as for the amperometric sensors, requiring just a simple set of electrodes with the necessary surface modification to make them selective towards the ion of interest [57]. The measurements here are done at zero current condition (open circuit), by comparing the potential of the working electrode with a reference electrode and quantifying its changes with the presence of the target ions. A reported example made use of platinum electrodes modified with Mg^{2+} ionophores to study RNA–Mg^{2+} binding kinetics by measuring the concentration of magnesium ions [58]. A similar set of pneumatic valves comparable to those mentioned in the amperometric approach was used to study the reaction at different time points, with a time resolution in the milliseconds range and utilizing less than 20 μL for a single experiment.

The second potentiometric sensor type (FETs) consists of semiconductor channels whose switching voltage depends on the content of the surrounding ionic species, providing a highly sensitive way to measure surface potential changes [59]. Analysis of droplets with FETs (**Figure 6b.ii**) was proposed for the first time in 2016 using silicon nanowires as semiconductor channel [41]. Silicon nanowires are excellent candidates for sensing in microfluidics, with ultrasensitivity and CMOS compatibility. First, the authors probed the content of all droplets up to 10 Hz resolving pH and ionic strength values through measured variations of the current through the nanowires. They observed that droplets required a minimum length equal to the linear dimensions of the sensor. The gate potential was influenced by the oil–water interface using short droplets, which could be useful to detect interfacial charges. As proof-of-concept of a biological assay, the activity of the glucose oxidase enzyme was monitored by measuring the produced acidification. The enzyme activity was monitored in parallel with an integrated optical setup with fiber optics and a portable spectrophotometer, providing such dual detection for the first time. For this, the enzyme reaction was coupled to a second one consisting of horseradish peroxidase which reduced the produced hydrogen peroxide while oxidizing the liquid colorimetric substrate 3,3′, 5,5′-tetramethylbenzidine. In a following work, the same group demonstrated that the pH change is not a requirement for the monitoring of enzymatic reactions in droplets [60]. Here, they encapsulated β-galactosidase, whose activity could be monitored due to the different ionic content after the reaction. Some authors observed that extended exposure of basic pH could degrade the sensitivity of droplet sensing with FETs due to degradation of the gate oxide [61], which would require further investigation of more suitable materials.

4. Droplet-based micro-magnetofluidics

While the detection of electrochemical methods like ampere- and potentiometric methodologies are based on alterations of electric conductivities of liquid phases in their surroundings, the detection of further physical properties, not directly visible by mentioned technologies, give rise to additional functionalities in microfluidic

μTAS applications. One prominent example depicts the research field of micro-magnetofluidics, the combination of microfluidics and magnetism, where the detection of magnetic fields of magnetically active or responsive liquids or magnetic species, demonstrate high potential in various applications. In this research field, one distinguishes between fluid control and manipulation in open channels and confined channels. Here, the utilization, manipulation and detection of magnetic species like magnetic nano- and microparticles in micro-magnetofluidics demonstrate big potential especially in biological, chemical and medical analysis. These species can act as carriers for biological and biochemical markers and molecules, act as immobilization bases and markers for quantitative detection and analysis. In this section, the closed-channel micro-magnetofluidics will be in focus. Further information about open channel micro-magnetofluidics, or digital micro-magnetofluidics, can be found in refs. [62–64]. Micro-magnetofluidics represents an active research field in the last two decades, facilitating various microfluidic procedures like mixing, particle focusing, stream manipulation, droplet generation, pumping and cell sorting [65, 66]. A big step in improvement of these systems as μTAS were first reports in 2005 about integration of micro-scale magnetoresistive sensors in microfluidic channels for analysis of passing magnetic liquids, *e.g.* magnetic micro [67]-and nanoparticles [68]. The working principle of magnetoresistive (MR) sensors is based on the change of their electric resistance by external magnetic fields. Especially in μTAS applications, in order to achieve maximum information output, vast amounts of relevant data points are collected due to high-throughput analysis at high sampling rates. To tackle this challenge of big data treatment, machine-learning algorithms are on the rise in current μTAS sensor applications and examples on this integration will be addressed in the section.

In the following, four types of magnetic field sensors and their microfluidic integration will be covered, namely anisotropic magnetoresistive (AMR), giant magnetoresistive (GMR), tunnel magnetoresistance (TMR) and planar Hall effect (PHE) sensors. Typically, the magnetization in AMR sensors is located in plane and sensor elements are fabricated as thin 2D layers (**Figure 7a**). Although AMR effect is about 2% only, due to their simplicity in fabrication, low noise and robustness, AMR sensors are broadly used in different industrial applications. In contrary to AMR effect, which can be observed in single layers of ferromagnetic material, the giant magnetoresistive (GMR) effect is specific to multilayer stacks of alternating metallic ferromagnetic and nonmagnetic layers. The electric resistance of this stack is changed significantly (about 50%) when the magnetization of adjacent layers is changing from antiparallel (in zero magnetic field) to parallel in an applied magnetic field (**Figure 7b**). The tunnel magnetoresistance (TMR) describes stacks of ferromagnetic, antiferromagnetic and insulator materials and its electrical resistance changes when magnetization of free ferromagnetic layer switches from antiparallel to parallel. In

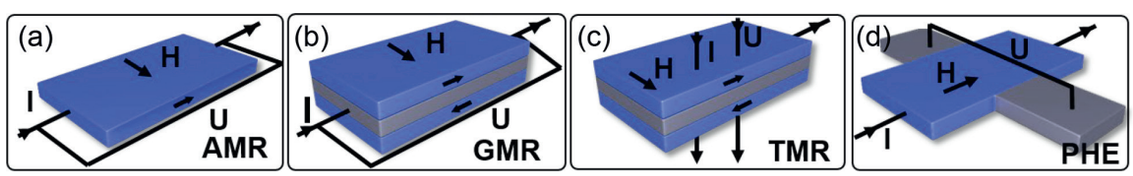

Figure 7.
(a) The anisotropic magnetoresistive effect (AMR) describes resistance alterations upon presence of magnetic fields based on intra-band electron scattering of ferromagnetic materials. (b) the giant magnetoresistive (GMR) effect in multilayer stacks of ferromagnetic and metallic layers. (c) Tunneling magnetoresistance is measured between the ferromagnetic layers across the isolation layer (cyan). (d) Planar hall effect (PHE) leads to variations of electrical conductivity by in-plane magnetic fields.

contrary to GMR, TMR sensors are measured perpendicular to the layer stack through the isolation layer (**Figure 7c**). The working principle of Planar Hall effect (PHE) sensors is based on anisotropic magnetoresistance [69] and spin Hall magnetoresistance [70] but, unlike AMR sensors, resistance alterations are measured transverse to the supplying electric current in electric anisotropically conducting thin films. When measuring the transverse voltage drop developed of this thin film, a planar Hall effect can be observed where the current will not flow collinearly with the voltage gradient. It experiences a transverse deflection towards the high conductivity axis, which results in an equilibrium transverse voltage drop (**Figure 7d**).

First reports date back to 2005, where principles and different types of MR sensors were presented and integrated into microfluidic systems, forming the base of micro-magnetofluidics, *i.e.* GMR [71–73], TMR sensors [67, 68], AMR sensors [74, 75] and PHE sensors [76]. Here, MR sensor were usually patterned using optical lithography on rigid substrates like silicon or glass with feature sizes in the μm range. First evaluations concentrated on detection of micron-sized particles. The next step in the evolution of MR sensors and micro-magnetofluidics was the addition of emulsion-based fluidic systems, *i.e.* microfluidic droplets. The first publications on the topic were focused on the detection and characterization of magnetic nanoparticle (MNP) based solutions, *i.e.* ferrofluids [77]. These superparamagnetic liquids broaden the field of application for micro-magnetofluidics, *e.g.* improved point-of-care diagnostics by better capture capabilities of MNPs for isolation of rare biomarkers and molecules, their ultrasensitive detection down to single-molecule sensitivity as well as drug discovery via remote control of individual droplets. With respect to MR sensor utilization for droplet-based micro-magnetofluidics, GMR sensors (spin valves and multilayers) are still one of the preferred sensor technology. Recent publications demonstrate advantages of PHE sensors due to their low limit of detection (down to 0.04 mg/cm^3 ferrofluid mass) and low noise (5 pT/$\sqrt{\text{Hz}}$ at 10 Hz) and thus their capability to measure outside of the channel geometry [78]. In the following, a detailed journey from the beginning of droplet-based magnetofluidics to present applications and integration with various types of MR sensors will be presented. The section is structured based on the microfluidic channel design to give an overview, starting from tubing-based channels (millifluidics), polymer-based channel systems (microfluidics) followed by insights and outlook to ultra-small channels in the nanometer regime (nanofluidics).

4.1 MR sensors in droplet-based millifluidics

As seen in Section 2.2, millifluidics depicts the technology of precise control and manipulation of liquids in tubings with inner diameters from about 500 μm, connected via commercially available connectors. Despite being not as versatile as microfluidics with respect to complex liquid manipulation, millifluidics offer the advantage of quick prototyping of fluidic setups, since lithographically approaches are not mandatory to build a functional fluidic setup. In combination with MR sensors, the distance between sensor and liquids have to be minimized since they cannot be integrated into the tubes, *e.g.* achievable via careful grinding of the tubings [78] or selection of tubings with low difference in their inner and outer diameters. One of the first reports of MR sensors and millifluidics was demonstrated by Melzer and coworkers in 2012 [79]. In this work, GMR sensors on elastic substrates were wrapped around the tubing to assure isotropic detection of magnetic objects (1 mm-sized clusters of FeNdB particles). This conjunction was possible and strongly benefited from the realization of stretchable magnetic

field sensors in 2011 [80]. In addition, these clusters were detected in macro-sized quasi-droplets (with a tubing inner diameter of 1.5 mm and droplet volumes in microliter regime). The first conjunction of droplet millifluidics and MR sensors was demonstrated by Lin and coworkers in 2013 [32], demonstrating a droplet analyzer based on the integration of GMR-based sensors into millifluidic tubings. Water-based ferrofluid droplets from 120 nL to 270 nL were fabricated using a commercial T-junction connectors and guided over a GMR sensor platform (**Figure 8a**). Hence, both volume of fabricated ferrofluid droplets could be detected by full width at half maximum (FWHM) of passing droplets over the GMR platform and concentration of the ferrofluid via the amplitude signal of the droplets, giving rise to a multiparametric analysis (in the spirit of flow cytometry) of passing ferrofluid droplets (**Figure 8b**). Furthermore, the detection and characterization of ferrofluid droplets was also demonstrated using PHE sensors (**Figure 8d** and **e**). Schütt et al. demonstrated the integration of tubing-based ferrofluid droplets onto PHE sensor enabling contact-less measurement in artificial magnetic fields (up to 5 mT) down to earth magnetic field without any external magnetization of the ferrofluid droplets [81]. Doing so, the sensitivity of droplet-based magnetofluidics could be increased by a factor of 100 (**Figure 8c–e**).

4.2 MR sensors in droplet-based microfluidics

The combination of MR sensors and droplet-based microfluidics is most commonly based on integration of MR sensors into the channels to achieve highest sensitivity. For microfluidic integration, MR sensors have to be as thin as possible not to disturb the laminar flow present in the channel. Practically, overall thickness of MR sensors (magnetically responsive layer, metallic supply and measurement electrodes as well as electrical isolation) should be optimally located in the nanometer regime, making these sensors composed either of 1D (nanowires) or of 2D (thin film) materials while general widths and lengths of individual components are located in the micrometer range. This

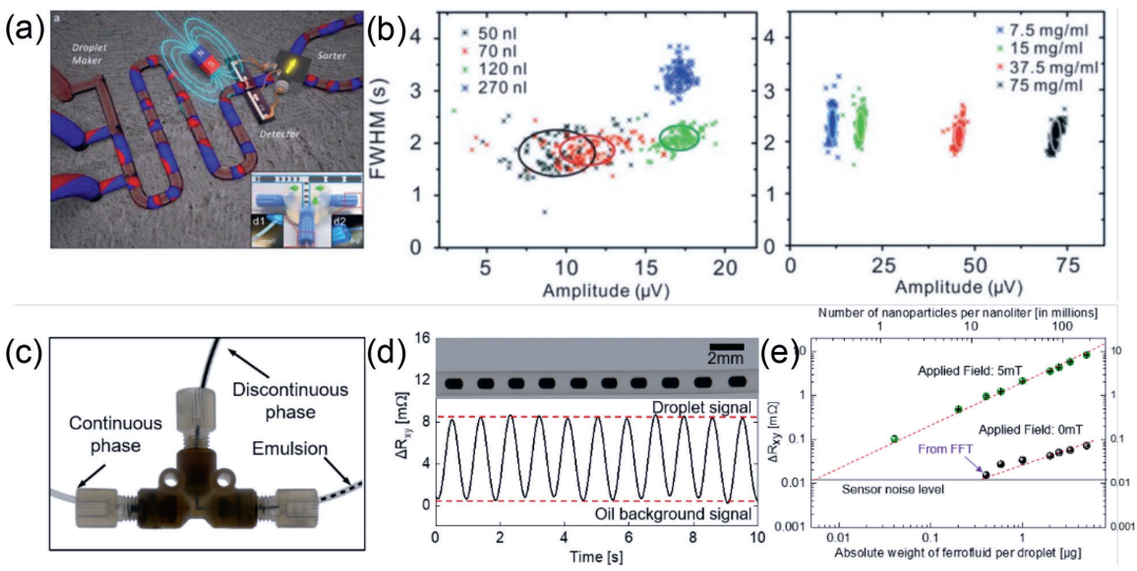

Figure 8.
Tracking and characterization of droplets in millifluidics using MR sensors (a) theoretical concept of ferrofluid droplet analyzer using GMR sensors enabling multiparametric analysis for (b) various droplet volumes (left) and ferrofluid concentrations (right) [32]. (c) Ferrofluid droplet generation in a commercial T-junction. (d) Detection of a ferrofluid droplet chain using PHE sensorics. (e) Investigations of limit of detection of the PHE sensor. Adapted with permission from ref. [81].

is achieved by elaborate patterning techniques such as micro-lithography by surface patterning and thin film deposition. Next to sensor dimensions, the compatibility of the sensors' substrate for microfluidic integration has to be given. Most preferably, substrates with hydroxyl groups (like silicon or glass) on their surface are preferred since channels can be easily sealed on. Next to rigid substrates, further functionalities are granted via alteration to flexible substrates. In the following paragraphs, several representative examples of MR sensor integration in droplet-based microfluidics are depicted.

The first conjunction of magnetism and droplet-based microfluidics using spin valve [82] and GMR [83] sensors in microfluidic channels were established by Lin and coworkers in 2015 allowing tracking of ferrofluid nanoliter droplets with various concentrations (5 mg/mL – 7.5 mg/mL) and lengths (150–750 μm). Here, a sustainable application depicts microfluidic coding and decoding assays due to high sensitivities of MR sensors to passing ferrofluid droplets. The μTAS setup contained an encoding area (droplet formation), encoded droplet pool as well as decoding area (GMR sensor platform) [83]. To further demonstrate the potential in the biotechnological and medical context, ferrofluid droplets of different concentrations were mixed with fluorescent dyes, specific for penicillin giving rise to a multidimensional microfluidic barcode (magnetic and optical). (**Figure 9a**). This system was subsequently improved in terms of information output by introduction of different concentrations of ferrofluids, thus generating binary coding signals with droplet chains of different concentrations of ferrofluid. The principle was firstly demonstrated in 2016 [84], a droplet-based micro-magnetofluidic μTAS system was developed facilitating the

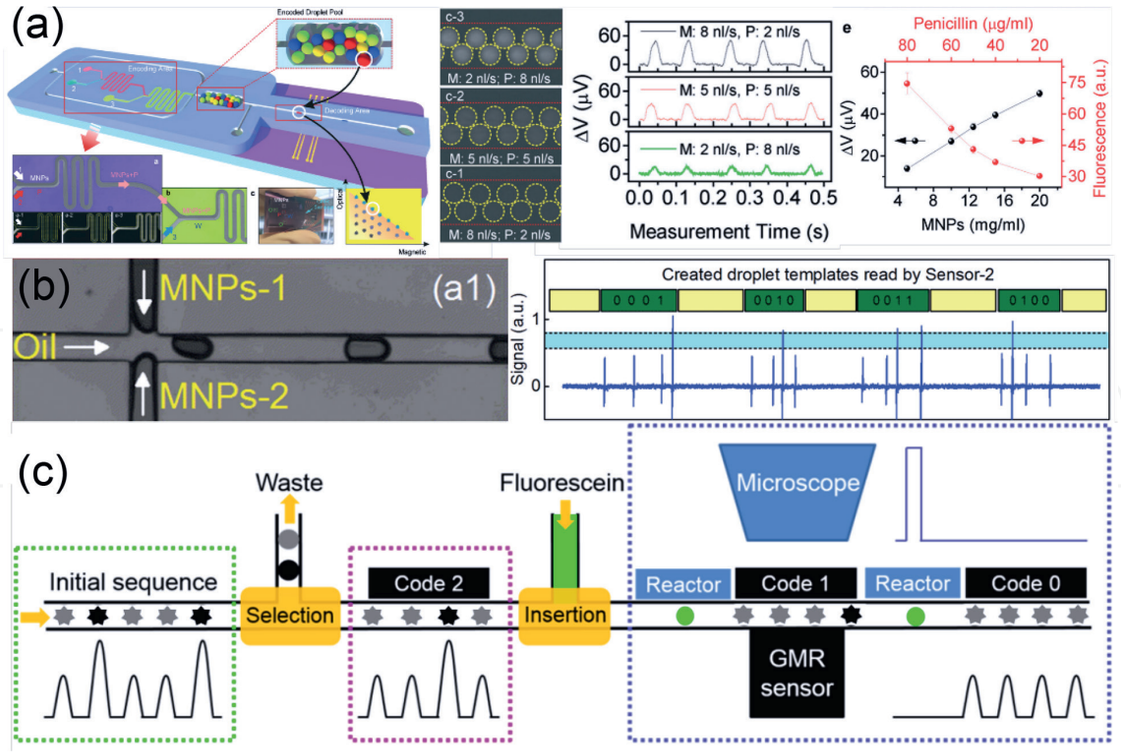

Figure 9.
Tracking and applications of droplets in microfluidics using MR sensors (a) droplet-based magnetofluidic platform with magnetic decoding using GMR sensors. Magnetic decoding (right side, M) was coupled with fluorescent decoding using fluorophore-coupled penicillin (right side, P) [83] (b) droplet-based micro-magnetofluidic μTAS system for generation of microfluidic ferrofluid droplet codes with downstream decoding region (GMR platform). Codes consisted of four adjacent ferrofluid droplets and two ferrofluid concentrations [84]. (c) Improvement of (de-)coding platform using ferrofluid droplets and GMR sensors by determination of the code generation, code starting point and final readout. Adapted with permission from ref. [85].

formation of binary droplet codes using four adjacent ferrofluid droplets via two different ferrofluid concentrations (**Figure 9b**). Downstream of the verification sensor, a GMR sensor, the droplets were further polymerized using a standard alginate and Ca^{2+} polymerization reaction to maintain their code for long time storage. Finally, to tackle the challenge of decoding, code spacing and interpretation, Wong et al. developed a complementary system allowing precise determination of the code generation, code starting point via introduction of a fluorescent marker droplet at each code beginning and a final readout (**Figure 9c**) [85]. In the spirit of ultra-sensitive high-throughput detection in micro- and millifluidics, state-of-the-art biosensors based on micro- and nanomaterials lead inevitably to big amounts of data points. Here, the trend of the analysis shifts from manual to automatic methods to meet the challenges of complex signal identification and interpretation. In this regard, machine-learning algorithms (MLAs) are starting to play a major role. To optimize the data interpretations from Lin's work of multiparametric detection and characterization of droplets [83], a MLA based on supervised discriminant analysis was developed. Another example is the work of Schütt and coworkers in 2020 analyzed by an MLA based on unsupervised k-means clustering algorithm [81].

In general, rigid substrates like silicon wafers or glass slides are preferred as substrates for microfluidic μTAS, since channel materials can be easily integrated onto these substrates. However, the transfer to flexible polymer-based substrates offers several advantages, for instance an increase of flexibility and efficiency in (bio-)detection. Furthermore, flexible substrate materials potentially lower material costs and weight compared to silicon-based substrates. Weight reduction helps to lower transportation costs, which is perfectly in line with the spirit of point-of-care systems. The utilization of polymer materials greatly increases the possibilities to fabricate biocompatible biosensors for potential applications in vivo. The fusion of magnetic detection technologies was transferred to flexible substrates by Lin and coworkers (**Figure 10**) [86]. Here, high performance GMR sensors were integrated between two flexible polymer layers patterned with a microfluidic channel system to create ferrofluid droplets, guided over the sensing structure. While the limit of detection was found at around 4 mg/mL, thereby allowing multiparametric detection of magnetic contents and droplet sizes, the whole device

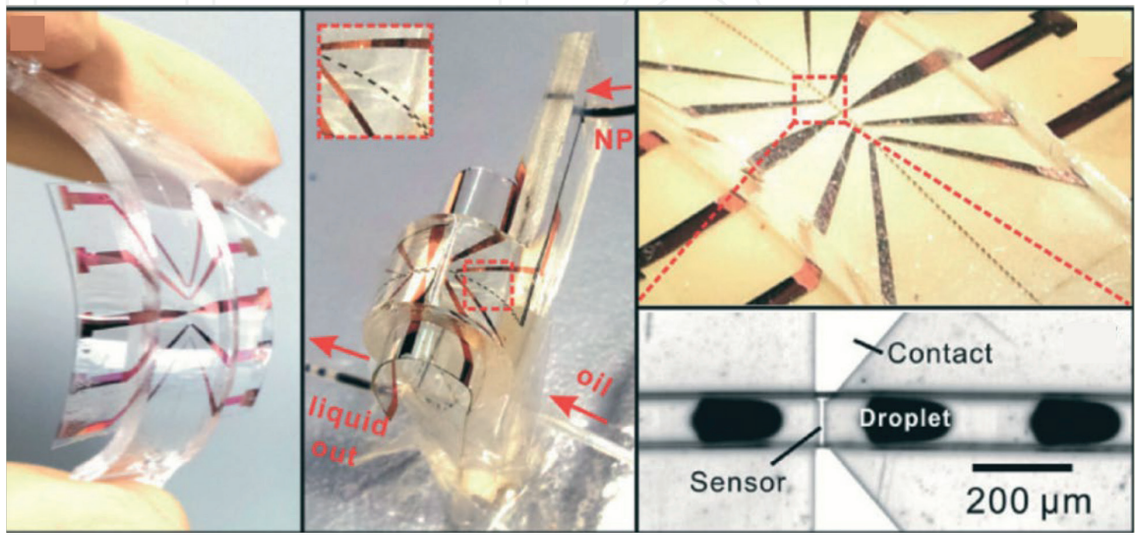

Figure 10.
Utilization of flexible substrates: Photographs for the droplet-based micro-magnetofluidic platform with integrated flexible GMR sensors. Adapted with permission from ref. [86].

could be bent to a radius of 2 mm, maintaining full functionality and performance. The utilization of inexpensive materials give rise to development of μTAS even in resource-limited environment and increases the possibilities of applications, *e.g.* wearable health monitoring, point-of-care testing and implantable solutions by increased flexibility in material utilization with respect to biocompability. As mentioned above, the alternative route of flexible substrates was also conceptionally explored in tubing-based millifluidics [79] demonstrating advantages of isotropic detection in fluidic channels. While the integration in tubing-based systems is rather simple via wrapping the flexible substrate around the tubing, the approach in microfluidics is more complex.

4.3 MR sensors in droplet-based nanofluidics

The research field of nanofluidics describes the miniaturization of microfluidics to the nanometer regime, dealing with channel sizes typically below 1 μm. In the last decade, droplet-based nanofluidics received high interest, since droplet volumes in these channels can be scaled down to even atto- to femtoliter volumes. These ultra-small volumes allow capture, isolation and synthesis of single molecules and are of high interest to understand biological or chemical processes, *e.g.* in enzymatic and kinetic activities. Common nanofluidics channel material is, as for microfluidics, PDMS where the typical droplet length in these channels is located from 2 μm – 3 μm. Leman and coworkers [87] demonstrated the implementation of a PCR reaction in femtoliter droplets in nano-channels while the volumes can be further reduced to stable attoliter droplets in nanofluidics channels [88] (**Figure 11a**).

To our knowledge, MR sensor have not yet implemented into these ultra-small channels. However, they have potential for analysis of the attoliter-droplets due to their ultra-high sensing capabilities in the pico-Tesla (pT) regime. The smallest measured droplet size until now, depict picoliter droplets of ferrofluid in PDMS-based microfluidic channels. Firstly detected by Tondra et al. by integration of GMR sensors into microfluidic channels at small cross-sections (13 × 18 μm) [89]. The sensor platform where 4 spin-valve GMR sensors (20 × 4 μm^2) with 2 sensing GMRs inside and 2 reference GMRs outside the channel in a Wheatstone bridge configuration (**Figure 11b**). For integration of MR sensors in nanometer-sized channels, drastic downscaling of sensors has to be achieved at or below the standard sizes of the droplets in the single μm regime.

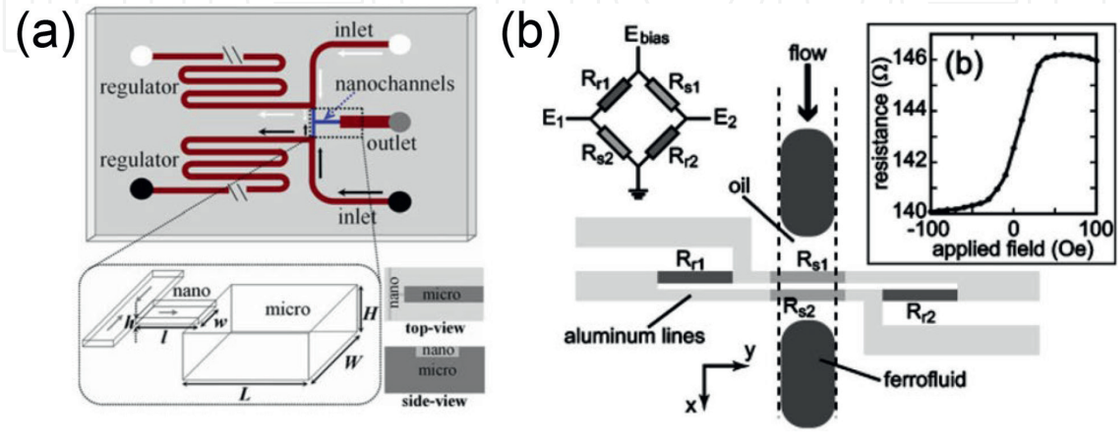

Figure 11.
(a) Conceptual scheme of a droplet generating T-junction in nanochannels. Adapted with permission from ref. [88]. (b) Integration of GMR sensors into microfluidic channels (13 × 18 μm). The droplet size was located in the picoliter regime. Adapted with permission from ref. [89].

Still, since the droplets are in the micrometer regime, elaborate patterning techniques using optical lithography are suitable. In future, this conjunction can greatly affect the analysis of ultra-small volumes of liquids.

5. Conclusions

Droplet microfluidics as a technique for biosensing applications represents an innovative and versatile approach as it offers high throughput, and the possibility to work with a multitude of reagents and samples using very low volumes. This technique also enhances the diffusion and mixing of species and reduces cross-contamination between different confined reactors and experiments. Likewise, droplet microfluidics has been shown for a variety of applications, ranging from the production of micro- and nanomaterials, to the performance of chemical reactions, microbiology and cellular analysis, including steps like sample preconcentration, incubation, mixing, and separation. However, most of these processes have been monitored via optical microscopy. Therefore, in this chapter we have highlighted the possibility to integrate other transducers that on one side enable the further miniaturization of droplet-microfluidic based systems, and on the other side provide new means of characterizing the processes taking place inside such tiny reactors. The range of characteristics and parameters that one can analyze in a sample are broader than just analyzing their morphology, size and color, for instance by determining their capacitance, inductance, magnetoresistance, which provide insights about for example interfacial charges, cell membrane proteins, expressed biomarkers, among others. Here, we also discuss the challenges in doing such an integration as we are talking about a multiphase system, where typically oil and aqueous solutions are employed to produce the droplets. Thus, if such phase change interferes with the active surface of the sensing device, new ways of integration and/or surface treatments or encapsulations should be taken into consideration. Finally, we also mention the requirements and solutions when moving across scales from mili-, to micro and nanofluidics, which would significantly affect the production and treatment of such devices and the further integration and miniaturization of sensors for a determined application. In summary, the combination of droplet microfluidics with different readout techniques would increase the applicability and monitoring efficiency of different chemical and biological processes at different scales. It would also allow multiparametric detection, combining the advantages for instance of electrical sensors with miniaturized optical readouts and new computational tools like machine learning and artificial intelligence which facilitate the interpretation and analysis of the high-dense data set obtained with such kind of platforms.

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Conflict of interest

The authors declare no conflict of interest.

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