Synchrotron radiation infrared microspectroscopy of single living cells in microfluidic devices: advantages, disadvantages and future perspectives

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Synchrotron radiation infrared microspectroscopy of single living cells in microfluidic devices: advantages, disadvantages and future perspectives

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Abstract. The possibility to fully exploit the diagnostic capabilities of SR-IRMS for studying single living cells under physiological conditions is limited by several constrains. First of all, the technology for manufacturing materials transparent to both IR and visible light is quite immature, limiting the design of fluidic devices to simple demountable liquid cells. In addition, the water spectral features become prominent in the Mid IR, hiding several cellular bands and therefore limiting the diagnostic capabilities of SR-IRMS. The overcoming of the so called "water absorption barrier" requires the improvement of the protocols for the compensation of buffer spectral contributions, a goal that can be achieved also advancing the quality of IR-suitable fluidic devices. In this paper, the technical solutions employed for microfabricating completely sealed IR-visible transparent fluidic devices for living cell analysis will be presented. Several examples of the results obtained in the study of living U937 monocytes subjected to different stimuli will be selected for highlighting both the advantages and the disadvantages offered by our approach for cellular biology.

1. Introduction

In the post-genomic era many attentions have been devoted to the understanding of structure and dynamics of macromolecules, considering their multiple complex interactions as a part of a biological system, following a multidisciplinary approach known as system biology. It is therefore not surprising that in recent years several Nobel Prizes have been awarded to scientists who have further developed well established analytical techniques for the study of biological systems including mass spectrometry

(Chemistry 2002 John B. Fenn and Koichi Tanaka), NMR spectroscopy (Chemistry 2002, Kurt Wüthrich), magnetic resonance imaging (Physiology or Medicine 2003, Paul C. Lauterbur and Sir Peter Mansfield) and fluorescence microscopy (Chemistry 2008, Osamu Shimomura, Martin Chalfie and Roger Y. Tsien). This testifies that the development of new analytical techniques as well as the more effective exploitation of existing methods is crucial for obtaining a better picture of the details of bio-molecules and/or bio-processes, part of the entangled network of the biological organization. However, the complexity of the biological systems is such that no single experimental method can provide information on all aspects of molecular structure and function, first of all because no one satisfies all the bioscience requirements, in short a label-free single-cell based assay sensitive, selective, working under physiological conditions (or better *in vivo*), no damaging and having nano-scale spatial and temporal resolution, or even better. InfraRed MicroSpectroscopy (IRMS) of cellular sample is not a single-molecule detection technique but it is a label-free method, very sensitive and not damaging, also if operated with Synchrotron Radiation (SR) [1], whose brilliance allows achieving diffraction-limited spatial resolution, which is of few microns in the MIR [2]. SR-IRMS has been largely employed for the characterization of individual single cells [3], also with sub-cellular spatial resolution, but it has been almost exclusively limited to fixed or dried cells, due to several limitations imposed by aqueous media in both sampling techniques and data analysis. These limitations can be roughly classified into technical and spectroscopic.

From a technical point of view, IR suitable materials impose many constrains on the fabrication of proper fluidic devices for living cell analysis. Recently published works on the study of living species under physiological conditions have been carried out by using commercial or custom made demountable liquid cells, produced spacing apart two optical windows by using a plastic spacer [4-6]. This approach does not ensure any design flexibility, limiting the fluidic design to a "pond" for cells, preventing any possibility to both manufacture fluidic elements, such as microchannels or microwells, and integrate other device functionalities, in a way similar to more complex Lab-On-a-Chips (LOCs). Moreover, the optical path in demountable cells cannot be carefully controlled: it is not perfectly reproducible from an experiment to another, and possibly differs in distinct device regions. All these aspects make even more difficult the overcoming of the so called "water absorption barrier".

When collecting the microspectrum of a living cell, assumed of spherical shape, setting the knife-edge apertures of Vis-IR microscope in order to fit the cell diameter, more than 80% of the sampled volume is constituted by water, ~48% of extra-cellular and ~36% of intracellular water, considering the cellular density close to 1 Kg/m^3 . It is therefore not surprising that the IR microspectrum of a living cell is dominated by water features (see Figure 1a), looking quite unusual if compared to standard fixed cell spectrum (see Figure 1b). The latter reflects the composition of a dried cell, made of ~ 50% of protein, ~ 15% of carbohydrates, ~ 15% of nucleic acids, ~ 10% of lipids and ~ 15% other molecules for an eukaryotic animal cell [7]. When the same cell is measured under physiological conditions, stretching bands of amine and hydroxyl moieties, mainly of proteins and carbohydrates, are completely hidden by the symmetric (ν_1) and asymmetric (ν_3) stretching bands of water O-H while bands characteristic of nucleic acids and carbohydrates falling below 1400 cm^{-1} as well as symmetric and asymmetric methyl and methylene stretching bands between 3000 and 2800 cm^{-1} are still discernable. For what concerns proteins, the water bending band centred at ~1643 cm^{-1} (ν_3) completely hides Amide I band and partially the Amide II, limiting the diagnostic capabilities of IRMS, especially toward cellular proteins, unless the buffer contribution to IR single-cell microspectra is compensated.

In this paper, both technical and spectroscopic aspects of single cell measurements under physiological conditions with SR-IRMS will be addressed. Selected experiments done over the past years at SISSI beamline (Synchrotron Infrared Source for Spectroscopy and Imaging) at Elettra [8] will be explained for highlighting advantages, disadvantages and future perspectives toward the upgrade of SR-IRMS to a mature label-free single cell-based assay.

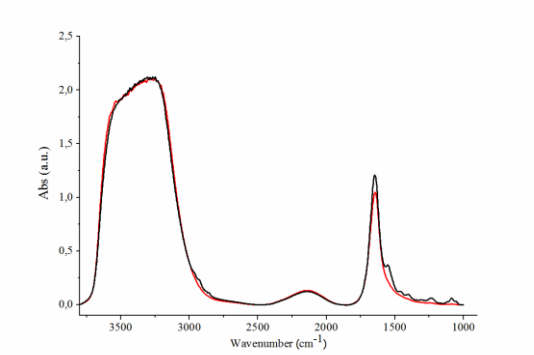

Figure 1a. SR-IR microspectra of an individual living U937 monocyte and buffer, black and red lines respectively.

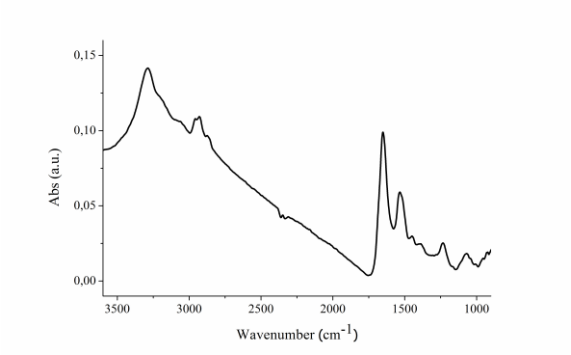

Figure 1b. SR-IR microspectrum of an individual U937 monocyte formalin-fixed.

2. Experimental session

2.1. Fluidic devices fabrication

In principle any design can be obtained by lithography, with spatial resolution of the details and fabrication time imposed by the type of lithographic approach chosen. Photolithography (also known as UV-lithography) allows to easily achieve micrometric resolution, perfectly matching the lateral resolution of SR-IRMS; it is fast and cheap, and therefore it was our first choice. As an example of device fabrication, we will detail the protocol used for manufacturing the device shown in Figure 2a. XARP 3100/10 (All Resist GmbH, DE) photoresist was spun onto a 2 mm thick CaF_2 optical window 3 cm in diameter. The achieved resist thickness was 7,5 microns. The resist layer was pre-baked at 85°C for 3 min and then exposed to UV light (I-line of Hg arc lamp, 365 nm) through a chromium-quartz mask at a dose of 90-100 mJ/cm^2. The pattern was then obtained by development in AR 300-26 for 45 s, followed by rinsing in deionized water and drying by blowing nitrogen. The device was then closed with a second CaF_2 window 1 mm thick through a thermo-mechanical process which exploits the residual solvent trapped inside the resist as a consequence of a milder resist pre-bake with respect to conventional parameters. The two windows were brought in intimate contact by a hydraulic press whose plates were heated to 65°C. At this temperature, a pressure of 5 bar was applied for 5 minutes, then the system was cooled down to room temperature; finally, the hereby sealed device was released. On the bottom window, 4 holes have been drilled before the lithographic process, in order to allow the connection with the external world assured by 4 L-shaped channels produced into the plastic holder shown in Figure 2c. The device thermalization is done by inserting the assembly in a cylindrical metallic heater. The temperature is set to 37 °C by a PID electronic controller which reads the temperature measured with a K thermocouple fixed onto the heater.

2.2. Data acquisition and analysis

Just before measurements, U937 monocytes were removed from the culture medium, that was exchanged with physiological solutions, and the cell suspension was injected inside the fluidic device using a syringe pump. IR microspectra of single living U937 monocytes have been acquired at 10x10 μm at SISSI beamline averaging 256 scans. A buffer spectrum was also acquired with the same parameters in a point close to the cell. Air background was collected within the appropriate device compartment. The “pure” cellular spectrum was then retrieved from the raw cell one by subtracting the buffer spectrum applying a scaling factor of 1 (Method 1) or adjusting it in order to match the water

combination band of the two centered at ~2125 cm^{-1} (Method 2). In respect to Method 2, a MATLAB algorithm was written that minimizes the residual area between raw cell and buffer microspectra in the 2500-1850 cm^{-1} spectral region.

a

Figure 2a. Actual device obtained by lithographing XARP resist on CaF_2 window. It is made by two concentric chambers separated by a porous septum.

b

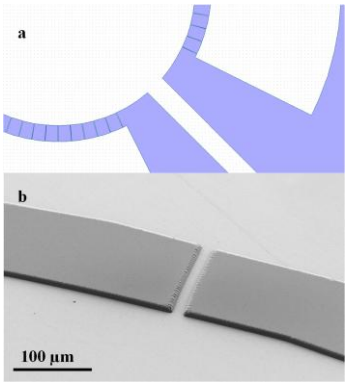

Figure 2b. Details of the device. In the upper figure the CAD lay-out of the porous septum with the inner chamber inlet; the lower is a SEM micrograph of the actual septum.

c

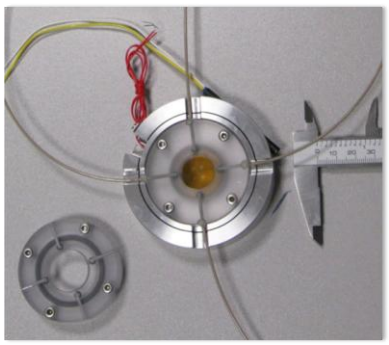

Figure 2c. Assembly of the micro-fluidic chip inside the external metallic heater and the fluidic connection system.

3. Results and discussion

3.1. Microfabrication of fully-sealed microfluidic devices

Biological MicroElectroMechanical Systems (Bio-MEMS) are analytical platforms where biological matter, from single molecules to entire living organisms such as bacteria, is manipulated in order to measure its response to different kind of stimuli [9, 10]. They are devices that comprise a wide variety of miniaturized structures, including moving parts (cantilevers, valves, and switches), static elements (channels, wells and porous sects), chemically functionalized surfaces (for promoting cell adhesion, as an example) and electrical components (resistors, T- and pH-probes). Bio-MEMS have revolutionized biological and medical science by reducing the reagent volumes for analysis and related costs, by shortening the time for analysis due to the higher effective concentrations reached within the devices, by increasing the throughput of many investigation techniques thanks to the parallelization of the detection and for many other reasons. However, such novelty did not impact IRMS and the reason has to be ascribed to the material requirements that have to be satisfied for producing IR-suitable bio-MEMS. Among the materials transparent to both Mid IR and visible light, the ones suitable for being microfabricated should be also biocompatible, economic and easily workable; however, none of the most common ones fulfil all the requirements. Fused silica, that satisfies all the Bio-MEMS requirements, has very poor transmission properties below 2500 cm^{-1}, while the good biological, optical and fabrication properties of diamond are paid at great cost. ZnSe is toxic for cellular samples [11] and also poorly transparent to visible light, while BaF_2 is quite soluble in water and therefore not suitable for our purposes. Calcium fluoride is matching almost all the requirements, except for the manufacturability. Fragile and with low thermal conductivity, CaF_2

cannot be easily "subdued" to microfabrication rules and the work done by our group was to optimize all the fabrication steps for produce a fully-sealed microfluidic device suitable for IR analysis, from the resist choice to the sealing step, included the photolithographic layout definition. It is beyond the aim of this paper to detail the optimization of fabrication protocols, briefly described in the experimental session and already detailed elsewhere [12] while we would stress on the advantages offered by microfluidic devices for IRMS analysis of living cells. The major improvement brought by the micro-approach is the design flexibility intrinsic in the method. Just as an example, the layout of one of the fluidic devices employed for the experiments presented in subsection 3.2 is reported in Figure 2a. It is made by two concentric chambers, separated by a porous septum, both connected with the external world via inlet-outlet macro-channels (see Figure 2b for details). The inner chamber accommodates the cells while the second is a solution reservoir where fresh physiological solution or chemical agents can be injected in, that will diffuse towards the cells while cell metabolites will diffuse out by osmosis. Syringe pumps are used for supplying both liquid and cell suspension, maintained at a constant temperature by a heater made by a resistor tape embedded into a stainless steel holder (see Figure 2c). The fluidic device accommodate also 4 isolated measurement points where air spectra can be collected without disassembling the device, as required by demountable liquid cells. Moreover, in respect to demountable liquid cell, the control of the device height is much more accurate and reproducible, both aspects that impact on the accuracy of water subtraction, as will be detailed later on.

For increasing the device throughput in the nucleic acid – carbohydrate spectral region, we implemented a sputtering process for deposit very thin silicon layers (10-20 nm) on top of BaF_2 optical windows, in order to avoid its dissolution in water with consequent cytotoxic effects on cellular sample and decreased IR-Vis transparency. Moreover, the silicon interface has two other major advantages:

1- It allows to employ more conventional resist, such as SU-8 or PMMA, as well as better established fabrication protocols
2- It allows exploiting the silane chemistry for the functionalization of the surface directly in contact with cells, for better mimicking the physiological conditions.

3.2. SR-IRMS of single living U937 monocytes

By using CaF_2 microfluidic devices, we performed several experiments in order to monitor the biochemical response of U937 monocytes to different kind of stimuli, both mechanical and chemical. These experiments will be summarized in this subsection for highlighting both the advantages and the disadvantages of single-cell IRMS with SR under physiological conditions.

The crucial step in the pre-processing of IR microspectra of living cells is the compensation for water features, in order to disclose the cellular spectral details, and in particular the protein bands Amide I and Amide II. For achieving this goal, the water contribution has to be subtracted, following standardized criteria. On the problems related to the reproducibility of spectral subtraction of water for the determination of protein secondary structure from dilute protein solutions, many authors have already reported [13, 14]. The faster way, named Method 1 hereafter, is to ratio the aqueous protein single-beam spectrum against the blank buffer reference. This method bases on two major assumptions: *i*- there are not significant differences in pathlength between recording the solvent and the protein single channel; *ii*- the water spectrum contributes for the same amount to the protein mixture and the buffer. By choosing a buffer point close to the acquired cell, the former criterion is quite well satisfied while the latter assumption can be considered almost always satisfied only for dilute protein solutions. This is obviously not the case of cellular spectra: rationing the cell single channel over the buffer single channel produces a microspectrum clearly over-subtracted, as the one shown in figure 3a, where Amide I and Amide II have almost the same intensity. A second route (Method 2 hereafter) can be followed for the pre-processing of living cell microspectra, similarly to the one most commonly employed also for dilute protein solutions: recording a reference spectrum of the air inside the device before the single channel either of the cell or of the buffer, and to ratio each

single-beam spectrum against the most recent air-reference. At this point the "pure" cellular spectrum can be retrieve from the raw cell one by subtracting the buffer contribution. In this approach, the major source of uncertainty lies in the scaling factor to be applied. One possible criterion of choice, reported also by other author [15, 16], is the flatting of the spectral region between 1800 and 2500 cm^{-1}. This region is almost free from cellular spectral features and it is dominated by the water band centered at ~2125 cm^{-1}, combination of bending band (ν_2) and librational modes of water. The dependence on hydrogen bonding of water librational modes makes them sensitive to changes in liquid and solution structuring, and this represents the real insurmountable limit of this approach, at least at the actual stage of knowledge. Intracellular water structure and organization is quite complex and still debated [17] but for sure it greatly departs from the dilute solution state of the buffer medium. However, there are not other water reference bands that can be used, since all of them fall in spectral regions non cellular free. This implies that a countless degree of uncertainty is intrinsic in the technique and major efforts should be done for avoiding other source of errors. In this respect, microfabrication is fundamental in achieving more controllable conditions. Indeed, the possibility to use microfluidic devices containing dedicated wells for the collection of the air background is quite helpful, since an individual cell can be monitored for long time, even days, re-collecting background every wished time, without the need to open the device as in the case of demountable cells. The repeatability of Method 2 can also be improved in respect to the current approaches where the choice of the best scaling coefficient is carried out by using interactive graphic programs, therefore being user-biased. In this regard, we developed a MATLAB routine for minimizing the residual area between the combination band of cell and buffer microspectra by varying the scaling coefficient between 0.8-1. The developed automated algorithm was used for the pre-processing of data presented in the following.

It has to be highlight that the difficulties associated to data pre-processing for circumventing the water absorption barrier are partly compensated by the fact that the closer matching of the refractive indexes of cells [18, 19] and water [20, 21] with respect to air greatly suppress the Mie-type scattering phenomena that deeply affect microspectra of single fixed cells [22]. This can be easily appreciated by comparing Figure 1b with Figures 3a and 3b, that show the microspectra of the same single U937 cell obtained by applying Method 1 and 2 respectively.

a

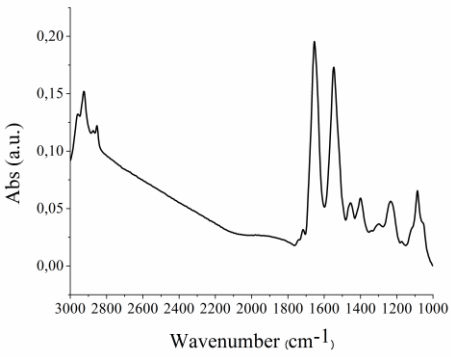

Figure 3a. SR-IR microspectrum of an individual living U937 monocyte obtained by applying the Method 1

b

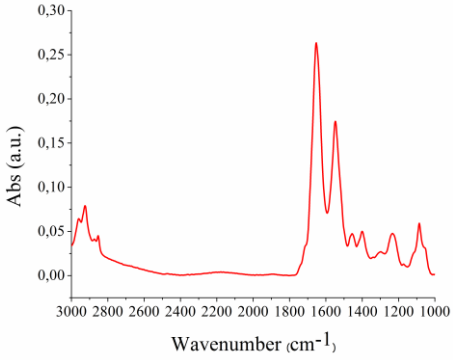

Figure 3b. SR-IR microspectrum of an individual living U937 monocyte obtained by applying the Method 2. Scaling factor determined in 0.924 via MATLAB routine

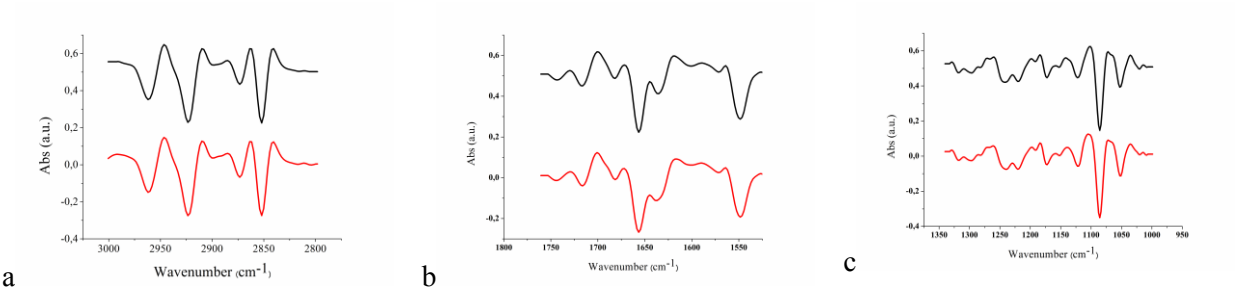

Figure 4a, b, c. Second derivatives of U937 microspectra (Savitzky-Golay algorithm, 13 smoothing point) obtained with Method 1 (black line) and 2 (red line) in the spectral regions a: 3000-2800 cm^{-1}, b: 1760-1480 cm^{-1} and c: 1350-1000 cm^{-1}.

Methods 1 and 2 are almost equivalent from a qualitative point of view, as evaluated by second derivative analysis (see figures 4a-c). No appreciable shifts in band position can be seen within the entire spectral range, with the exception of the broadening toward ~1645 cm^{-1} of the components of Amide I band in Method 2, accounting for the lower water subtraction than Method 1. On the contrary, many differences can be appreciated between the two approaches from a semi-quantitative point of view. As expected, the Method 1 is underestimating the integral intensity of both Amide I band (5.72 a.u. versus 11.87 a.u.) and, at a less extent, Amide II (5.97 a.u. versus 6.85 a.u.). This reflects in the lowering of Amide I over Amide II (AmI/AmII) ratio for the Method 1 (0.96) in respect to Method 2 (1,73). However, the AmI/AmII ratio retrieved with Method 2 is higher than ~1.5, commonly found in dried samples. This could account for structural water molecules, integral part of protein structure and fundamental for their biological activity [23, 24]. While the integral of DNA asymmetric band is equivalent by using the two methods, both lipid content and composition are method-sensitive: the total lipid content is overestimated (3.523 a.u. versus 2.906 a.u), while the methylene on methyl symmetric stretching ratio is underestimated (1.167 a.u. versus 1.285 a.u.). Similar results have been obtained for all the cellular samples investigated (data not shown) and compared with that coming from single cells fixed in formalin (data not shown), that is known to preserve both lipid content and composition. We found a better agreement between the formalin dataset and the one obtained with Method 2, which therefore we decided to systematically apply for the data pre-processing of living cell spectra.

Even if the application of the Method 2 is more time consuming, it offers some practical advantages beside the one previously highlighted. First of all, the acquired dataset can be always brought back to one obtained with Method 1, rationing the cell single channel over the buffer single channel, used as background. Secondly, from the buffer spectrum rationed over air background it is easier to check for the buffer cleanness. As a matter of fact, cells both excrete metabolites and chemical substances, in response to either external stimuli or naturally occurring events, for example the Extra Cellular Matrix (ECM), composed by fibrous proteins and glycosaminoglycans in the majority. The local accumulation of such cellular products can be quite high if the buffer medium is not properly exchanged, that is with a velocity sufficient for keeping cells healthy by providing fresh nutrients but lower enough for the completion of cellular events that need extracellular mediators, such as cell adhesion or duplication. Demountable liquid cells cannot be fluxed without the risk to push the investigated cell out from the field of view while extremely low fluxes do not guarantee an appropriate medium exchange. All these drawbacks can be solved by using a liquid cell with the design shown in Figure 2a, where a porous septum is dividing the cellular compartment from the medium reservoir that is therefore exchanged by slow diffusion.

It is evident that the exploitation of the advantages offered by microfabrication in the field of IRMS of living single cell with SR can open new and existing opportunities in monitoring the cell biochemistry under physiological conditions. We already demonstrated the possibility of following in real time the biochemical alterations undergone by living cells subjected to both chemical and mechanical stimuli. By stimulating U937 monocytes with fMLP, a synthetic peptide that mimics the activity of bacterially derived peptides with formylated N-terminal methionine groups, able to promote the monocyte extravasation for reaching the inflammation site, we monitored the synthesis of new adhesion proteins and the cell cytoskeleton rearrangement responsible for cell extravasation [25]. Similarly we monitored the response to U937 to mechanical deformation, confining them in fluidic devices of different thickness in order to achieve undeformed, deformed and heavily deformed conditions. We demonstrated that deeply deformed cells have a cellular biochemistry quite different from undeformed and slightly deformed ones, evidencing that the limit in fluidic device pathlength, imposed by the saturation of water bending band at ~9 microns, is in turn limiting the type of cells can be sampled to the ones that have an height lower that the pathlength constrain [26]. However, even if most of the circulating cells are usually larger than 8-9 microns, the majority of the adherent cells are not.

Finally, is has to be highlighted that a living cell never sleep. Therefore cell mapping, a quite long measurement, is not reliable since the risk is to start measuring an object and to finish with a completely different one. Therefore, fast SR chemical imaging is presently the more convenient approach for the fast collection of whole-cell cartograms with micrometric spatial resolution [27].

4. Conclusions

The extension of microfabrication concepts for producing fluidic devices where analyse living cells can promote the upgrade of SR-IRMS toward a mature label-free single cell-based assay. The design flexibility, one of the major advantages of the technique, allows performing innovative experiments never done before. We opened the road for the fabrication of CaF_2 devices, and we are testing new devices in BaF_2 silicon coated, that allow an easier access to nucleic acids bands and to tune the device surface properties by silane chemistry. However, even if we believe we did very big steps in optimizing fluidic for IRMS, some spectroscopic aspects have not be clarified yet and need further in-depth examinations. First of all, a standardized protocol has to be developed for the subtraction of water content from raw cell microspectra, in order to disclose Amide I band and give more reliable information also on cellular lipids. Microfabrication can help in pursuing this goal, but water is still imposing heavy limitations in cellular analysis, especially on cell dimensions. Finally, the advantage of monitor living cells in real time is imposing measurement times shorter than the time for completion of the phenomenon under investigation. In particular, fast imaging techniques are needed for pursuing living single cell analysis at sub-cellular spatial resolution, since a cell is always evolving.

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