In-vitro sub-cellular sampling of metabolites in biological samples.

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Abstract*— Chemical analysis of metabolites directly from cell samples at sub-cellular level in ambient conditions is essential to understand the biochemical pathways in these organisms. In order to achieve this goal we combine near-field ablation with mass spectrometry. As a first step towards the integration of near-field ablation with mass spectrometry, we will report preliminary results of the chemical analysis of *Arabidopsis Thaliana.

I. INTRODUCTION

Decoding the DNA is the first step in understanding the complex mechanisms within cells. However it does not specify the complex biochemical processes occurring in cells. Having a complete sequence of genomes is not sufficient to completely understand the complex biochemical mechanism occurring in cells [1]. To have a better understanding, it is also important to identify the various biomolecules that are expressed and how they are distributed in cells [2][3]. The spatial distribution of metabolites reflects the state of a cell and its relationship with the environment. The study of metabolites offers several advantages: metabolites are the end product of gene expressions which participate in the metabolic reactions required for the growth and normal functioning of the cell [4][5][6], they are closer to the cell's phenotype, and in humans the estimated number of different metabolites is roughly three orders of magnitude less than that of proteins [7][8][9]. The chemical analysis of metabolites directly from cell samples in ambient conditions can lead us to an understanding of disease mechanisms, the discovery of diagnostic biomarkers, and an ability to predict individual variation in drug response phenotypes [10][11].

Currently the identification and structural characterization of metabolites in tissue samples and large cells is possible through mass-spectrometry (MS)-based, soft-ionization techniques such as matrix-assisted laser desorption/ionization MS (MALDI). In MALDI the molecules of interest are embedded in a light absorbing matrix and their ionization is initiated by a laser beam, which also removes the molecules by ablation. Conventional MALDI requires extensive sample preparation and ionization is mediated with an ultraviolet (UV) laser in vacuum. Hence this cannot be used to investigate aqueous solution or *in-vitro* biochemical analysis. The development of infra-red (IR) MALDI has been extensively in the investigation of aqueous solutions [12]. Various techniques for ionization of molecules in ambient conditions have demonstrated the ability of ion production directly from samples without extensive sample preparation. Due to the strong absorption of water at 2.94μm wavelength the native water content in cells and tissues can serve as an efficient matrix to couple the laser energy into the cell samples. With these advances, the researchers are able to use the combination of atmospheric pressure (AP)-IR-MALDI to study the spatial distributions of analytes in a tissue section [13].

Although AP-IR-MALDI MS, has been used to analyze biomolecules from various plants, organs, and human body fluids [13][14][15], the diffraction limit restricts the focusable size of the laser to half the wavelength of the light used. For the IR method, available optics is not sufficient to focus to a spot size of less than 40μm. Even if these experimental limitations can be overcome, the Rayleigh criterion imposes a physical limit, that the size of the laser spot cannot be reduced further than $\lambda/2$=1.5μm for the wavelength of 3μm. In order to probe the role of metabolites in smaller eukaryotic cells, a method with a higher resolution is needed. In order to achieve high resolution, we combine near-field ablation with mass spectrometry. Using scanning probe microscopy (SPM) approaches, we break the diffraction limit and perform near-field ablations on cell samples. Our group has already studied near-field ablation and characterized the ablation mechanism in cell samples [16].

As a first step towards the integration of near-field ablation with mass spectrometry, we will report preliminary results of the chemical analysis of *Arabidopsis Thaliana* leaf sample. In order to map out the spatial distribution of the metabolites, it is necessary to have a spot size, small enough to be a fraction of the cell size. In these experiments we were able to achieve resolution as high as 1.5μm. A difficulty that arises when using such a small spot size is the relatively small amount of ablated material, which is on the order of only a few hundred zepto moles. Currently we are unable to directly analyze the ablated material with the mass spectrometer. To address this issue, we introduce an intermediate step of using nanopost arrays in combination with near-field ablation [17].

II. MATERIALS AND METHODS

A. *Samples for study*

Arabidopsis thaliana var. Col0 are sown onto soil, and kept 4 days at 4°C for stratification. The seeds are then transferred in growth chambers under long day conditions [18], and leaves from 8-weeks-old plants are detached for the analysis. The experiments described here are performed with an NSOM apparatus, modified for operation in the mid-IR region [19]. In all experiments, the mid-IR output of a Nd:YAG laser, coupled to a pumped optical parametric oscillator (OPO) (set to 2940 nm, 10 Hz, 5 ns pulse width) is used for ablation.

After ablation, which is performed in 0.5µl water drop, the ablated material in the water droplet is deposited onto a NAPA surface and air-dried. Laser desorption and ionization (LDI) MS of the ablated material is then performed using a high-resolution time-of-flight mass spectrometer (Axima CFR, Shimadzu-Kratos, Manchester, UK). Averaged spectra are obtained from 100 laser shots in positive reflectron mode using pulsed extraction switch delay of 100ns, 2.5kV extraction voltage, and 20kV accelerating voltage.

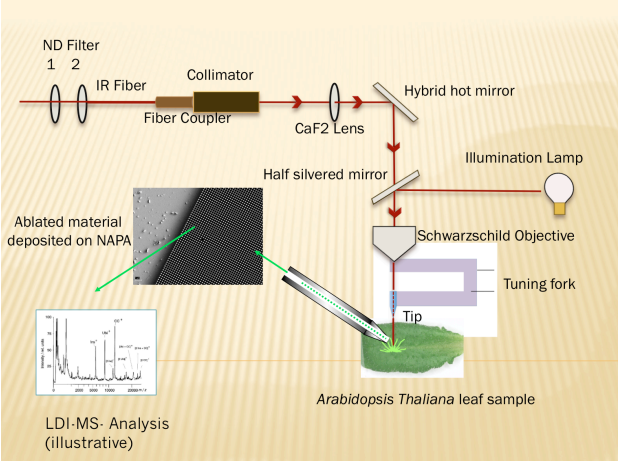

Figure 1. Schematic diagram of the experimental setup.

B. *Experimental set-up*

As shown in Fig.1, a 3µm wavelength laser is guided through the microscope, the light is then focused by the objective onto the back of the fiber tip (etched using modified tube etching technique) [20], which is attached to a tuning fork. We use tuning fork based AFM technique to bring the sample into the near-field region of the fiber tip. In this way, the illuminated area is defined by the size of the aperture, which enables the ablation of the material from spots smaller than the Abbe length [21] [22].

We perform the ablation in the water medium and after the ablation, the material is deposited on NAPA for LDI analysis. The NAPA chips are attached to a standard MALDI plate using a double-sided carbon tape and LDI-MS is performed on the material deposited on these chips.

III. RESULTS AND DISCUSSION

The ablation experiments on *Arabidopsis Thaliana* leaf sample is characterized using scanning electron microscopy (SEM). Figure 2 shows an SEM image of the ablation with a spot size of 2.5µm.

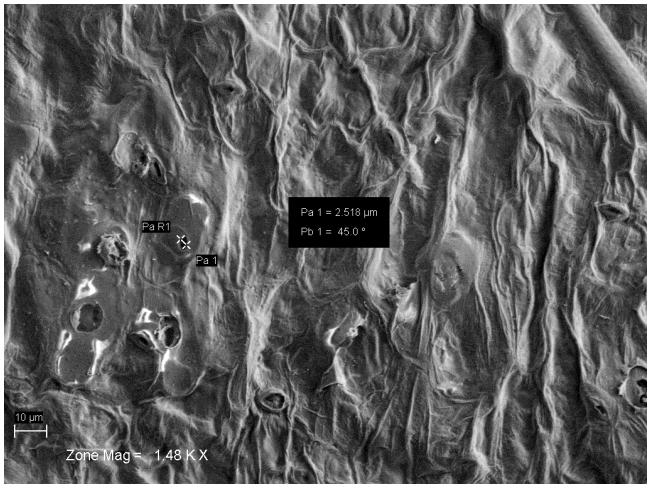

Figure 2. SEM image of the ablation spot on *Arabidopsis Thaliana* leaf sample.

Table1. Some of the metabolites detected in *Arabidopsis Thaliana* leaf sample after near-field ablation.

No.	***Assigned metabolite***	***Ion***	***Calcd m/z***	***Measrd m/z***	***Δm/z***
1	Ethylamine	[M+H]+	46.066	46.046	0.020
2	n-Butylamine	[M+H]+	74.097	74.071	0.019
3	Ethanolamine	[M+Na]+	84.043	84.066	0.023
4	Glycine	[M+Na]+	98.022	98.066	0.044
5	4-Aminobutyrate	[M+H]+	104.071	104.106	0.035
6	Pyroglutamate	[M+K]+	168.132	168.144	0.012
7	Hexose	[M+K]+	219.153	219.180	0.027

After verifying the successful ablation in water, LDI-MS analysis is performed on the ablated material. Taking the average leaf density of the order of 100µg/mm^3, the volume of the material that comes out of the ablation spot is of the order of 100 atto moles. We were able to identify as many as 16 metabolites from the near-field ablation experiment. The experiments were repeated 9 times. Ablation spots and the m/z values of few metabolites are shown in Table1. Figure 3 shows the mass spectrum as a comparison of two ablation

spots showing these metabolites. We observe that the relative intensity of these peaks varies for each metabolites at different spots. However, further investigation needs to be done regarding this variation in the intensity.

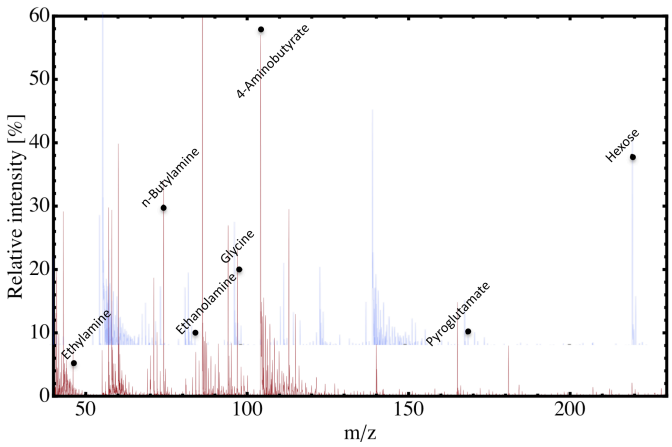

Figure3. Comparison of the MS spectrum for two different ablation spots. The black dots represent some of the metabolites.

In conclusion, we were able to identify different metabolites from *Arabidopsis Thaliana* leaf sample using near-field ablation experiment. We were able to show proof of principle that few hundred zepto moles of material can be detected in ambient conditions using near-field ablation coupled with MS. With this technique eventually we should be able to study the spatial distribution of different biomolecules in live cells and tissues with sub-micron resolution. This information is crucial in understanding various biochemical processes occurring in biological samples.

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