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MICROBEAM-COUPLED CAPILLARY ELECTROPHORESIS

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Within the first few microseconds following a charged particle traversal of a cell, numerous oxygen and nitrogen radicals are formed along the track. Presented here is a method, using capillary electrophoresis, for simultaneous measurement, within an individual cell, of specific reactive oxygen species, such as the superoxide radical ($O_2^{-\bullet}$) as well as the native and oxidised forms of glutathione, an ubiquitous anti-oxidant that assists the cell in coping with these species. Preliminary data are presented as well as plans for integrating this system into the charged particle microbeam at Columbia University.

INTRODUCTION

The field of single-cell analysis has been growing rapidly over the past few years[1], yet few studies incorporate a method of stimulating a single cell within a population followed by analysis of chemical components within that same cell. Typically, a population of cells is treated with a chemical stimulant, and the variability in response from individual cells is studied. Microbeam-coupled single-cell analysis techniques allow the researcher to both target an individual cell and analyse that cell, or others around it, and gain a better understanding of the network of responses initiated by the irradiation. At the Radiological Research Accelerator Facility (RARAF), coupling the microbeam irradiator to various single-cell analysis tools for studying gene expression and chemical changes within individual cells is currently being investigated. The first goal of this study is to quantify the formation of reactive oxygen and nitrogen species (ROS/RNS) and the oxidation of glutathione, one of the most abundant scavenger molecules.

Due to the small amount of material present in single cells, any chemical analysis technique used must be extremely sensitive. The nanolitre sample sizes used in capillary electrophoresis (CE)[2] make this technique suitable for small-scale biochemical applications and in particular for single-cell studies. CE provides good separation of analytes, based on electrophoretic mobility and short analysis times (typically a few minutes or less, from cell lysis to analyte quantification). The proposed introduction of single-cell CE and laser-induced fluorescence (LIF) technology at the RARAF microbeam endstation will allow rapid quantification of ROS and the oxidation state of glutathione. As experience is gained with this system, additional end points will be investigated.

CAPILLARY ELECTROPHORESIS

CE is a separation technique in which analytes migrate through a thin glass capillary under high electric field and are separated by their electrophoretic mobility differences. In CE, there are two superimposed flow modalities experienced by the analytes in the sample plug: (1) electrophoretic flow, responsible for separating the analytes by charge and Stokes radius[2], and (2) electroosmotic flow, which drives the buffer and analytes (regardless of polarity) towards the outlet of the capillary and the detector. The electroosmotic flow is driven by the interaction of electrolytic liquid with the capillary walls leading to the formation of a mobile Debye layer of charges and is much stronger than the electrophoretic flow, ensuring that all analytes will reach the detector. Migration time of individual analytes is given by

$$t = \frac{L^2}{\mu V} \tag{1}$$

where L is the length of the capillary, V is the applied voltage and μ is the electrophoretic mobility[3]; hence, faster separations may be achieved more readily by using short capillaries[4] than by using higher voltages.

FLUORESCENCE DETECTION

Coupled to CE, fluorescence detection provides extremely sensitive limits of detection, particularly when detecting brightly fluorescent molecules[5].

LIF requires either on-column or post-column excitation with a laser of appropriate wavelength, and efficient collection and measurement of fluorescence emission.

Using LIF, repeatable detection limits on the order of 70 molecules have been achieved[6]. This level of detection is enabled by the fact that each fluorescent molecule can cycle repeatedly between excitation and

Table 1. Examples of derivatizing agents for radiation-relevant species.

Species to be detected	Derivatizing agent	Peak excitation (nm)	Peak emission (nm)	Reference
Superoxide ($O_2^{-\bullet}$)	DHE, MitoSOX	400/500	600	(16, 17)
Hydrogen peroxide (H_2O_2)	CM-H_2DCF-DA	495	520	(15)
Peroxinitrite ($ONOO^-$)	HPF	499	515	(14)
Nitric oxide ($NO^\bullet$)	DAF-FM	495	515	(13)
Hydroxyl ($OH^\bullet$)	TA	315	425	(12)
'General' ROS	DHR123, DHE, MitoSOX	505	525	(9–11)
		500	600	
Glutathione (GSH, GSSG)	NDA, OPA	430	530	(18, 19)
		338	458	

emission, thus generating thousands of photons before undergoing photo-bleaching. The use of laser excitation allows this process to occur only while the molecule is within a limited detection region. Fluorescence measurements are less sensitive to many sources of optical background (e.g. scattered light)[7], since the observed emission spectra are shifted relative to the excitation wavelength and can be separated easily.

While fluorescent detection can be applied to peptides and proteins containing aromatic amino acids that can be detected directly using excitation wavelengths in the deep UV[8], most molecules do not natively fluoresce; for these molecules, fluorescence measurements require the use of fluorescent dyes (derivatization). For reactive species, many derivatizing agents are non-fluorescent analogues of standard dyes. These chemicals permeate the cell, nuclear and/or mitochondrial membranes and are oxidised by one or more types of reactive species into a fluorescent form[9–19]. Table 1 shows some examples of fluorogenic reagents used to observe various radiation-induced reactive species. Some agents (e.g. DHE, MitoSOX) have different fluorescent forms depending on the reactive species with which they interact[10, 11]; however, while these end products typically have similar emission spectra, which cannot be separated using standard imaging techniques, they have different mobility and can be separated spatially in a CE system and detected with <1000 molecule sensitivity[20].

In most cases, the derivatizing agent needs to be incorporated into the cell through the culture medium before irradiation. This 'stabilises' the short-lived (nanoseconds) ROS into long-lived (minutes to hours) fluorophores allowing measurements to be performed at long times after irradiation. As an example, Figure 1 shows derivatization of superoxide by DHR-123, resulting in Rhodamine-123.

Derivatization can also be used to detect other molecules. For example, the tripeptide glutathione (GSH), a ubiquitous anti-oxidant present in millimolar concentrations in cells, is one of the cell's primary defences against oxidative stress[21]. In the presence of ROS, similar to those generated by radiation, GSH neutralises the radicals, undergoing oxidation primarily to form a glutathione disulphide dimer (GSSG). The monomer and oxidised dimer can be separated by CE[18] and fluorescently detected. In this case, derivatization should be done following the separation[22] as GSSG does not have a free thiol group that can be labelled.

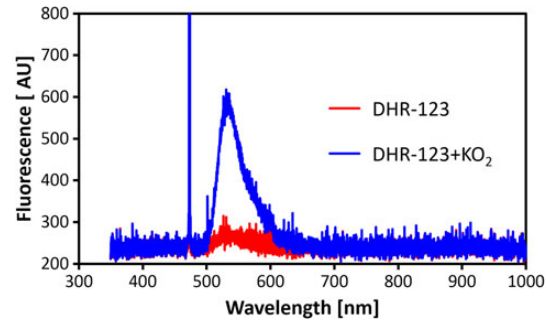

Figure 1. Fluorescence spectra measured using the RARAF breadboard LIF system of DHR-123 before (grey) and after (black) addition of KO_2, a superoxide generator. The spike at 473 nm is scattered light from the excitation laser.

COUPLING CE-LIF TO A MICROBEAM

A schematic of the proposed CE-LIF system is shown in Figure 2. A 30-cm-long fused silica capillary is brought in close proximity to the cell of interest[23] (Figure 2c). Immediately (or up to a few hours) following irradiation, a small amount of trypsin is dispensed onto the cell[24], followed by hydrodynamic[23] or electrokinetic[25] aspiration of the (released) cell into the capillary. The capillary is then removed from the dish, using an automated positioning system, and placed into a grounded vial containing background electrolyte (BGE).

Cell lysis and separation of analytes[23] are performed within the capillary, after application of high voltage between the grounded vial and the detection system.

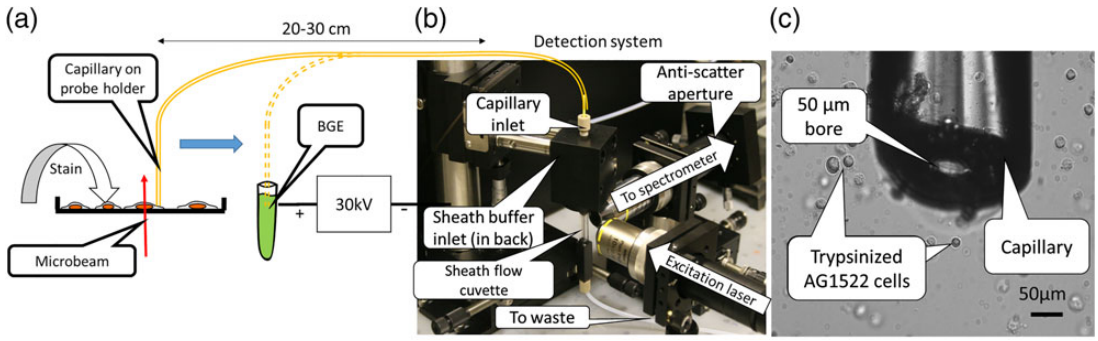

Figure 2. (**a**) Layout of the proposed microbeam-coupled CE-LIF system. After loading a cell into the capillary, the capillary tip is transferred to a grounded vial of BGE and negative high voltage is applied at the outlet of the sheath flow cuvette. (**b**) Prototype sheath flow LIF system constructed at RARAF. (**c**) Loading a single whole cell out of a cell suspension into a capillary at UIUC.

The outlet end of the capillary (Figure 2b) is inserted into a cuvette, in which sheath buffer flows in the same direction as the electroosmotic flow. The gravity-driven sheath flow matches or slightly exceeds the flow in the capillary, minimising turbulence and focussing the analyte stream, reducing background from the cuvette walls[5, 25]. A solid-state laser, matched to the optimal excitation of the derivatized analytes, is fibre-coupled to a 10× objective and focussed just below the capillary output. A second 10× objective collects the fluorescence light emitted at the intersection of the laser and analyte flow into an optical fibre connected to a spectrometer. The use of a spectrometer adds a second dimension of information to the analysis. A tandem measurement of the fluorescence emission spectrum allows resolution of analytes with similar migration times and more reliable identification of analytes than is available by migration time alone[26]. The amount of analyte, determined by the integral of the peak, can be calibrated by spiking cellular lysates with known amounts of analyte. Scattered light is rejected by an aperture placed between the objective and the fibre-optic or by an appropriate dichroic filter.

PRELIMINARY DATA

Using a prototype LIF system, at RARAF, consisting of a sheath flow cuvette[27] two Nikon CFI60 10× objectives, a 473-nm 50-mW solid-state laser (Laserglow Technologies, Toronto, Canada) and an SPM002 spectrometer (Photon Control, Burnaby, Canada), detection levels below 100 nM for both Glutathione-NDA and Rh-123 have been achieved. This concentration of Rh-123 corresponds to the ROS yield induced by 1 Gy of X rays, derivatized with an excess of DHR-123. A tenfold improvement in limits of detection is expected by upgrading the prototype LIF spectrometer to one with higher sensitivity and lower noise.

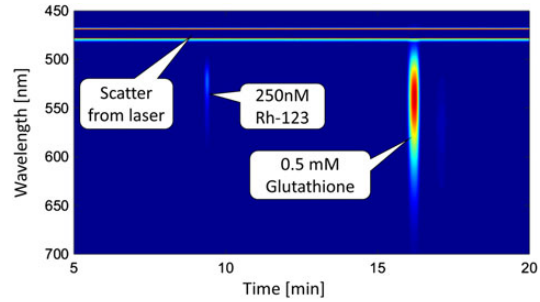

Figure 3. Wavelength-resolved electropherogram of 10 nl of a mixture of 0.5 mM glutathione derivatized with NDA and 250 nM of Rh-123. The Rh-123 signal-to-noise ratio is ~1500.

Using the existing CE-LIF system at the University of Illinois (UIUC)[27], preliminary studies on separation of ROS and GSH have been performed. Figure 3 shows a spectrum of a 10 nl mixture of 0.5 mM glutathione derivatized with NDA and 250 nM Rh-123, obtained using a 1-m-long fused silica capillary (ID 50 µm, OD 360 µm) and 50 mM borate buffer (pH 8.8) as BGE. The separation voltage was 21 kV.

These studies have allowed to evaluate potential system throughput. From Figure 3, detection times for Rh-123 and GSSG-NDA are 9 and 16 min, respectively, leading to a maximum throughput of 3–5 cells h^{-1}. These flow times can be significantly accelerated, without loss of separation[4], by reducing the capillary length. For example, a reduction in the capillary length from 1 m to 30 cm, without changing the separation voltage, will result in a ninefold decrease in migration time. Higher throughput is possible because, in contrast to CE separation of proteins/peptides, samples of

the present study contain only three to four detectable fluorescent species.

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