Analytica Chimica Acta xxx (2017) 1–9

Contents lists available at ScienceDirect

Analytica Chimica Acta

journal homepage: www.elsevier.com/locate/aca

Diffusion of cytokines in live lymph node tissue using microfluidic integrated optical imaging

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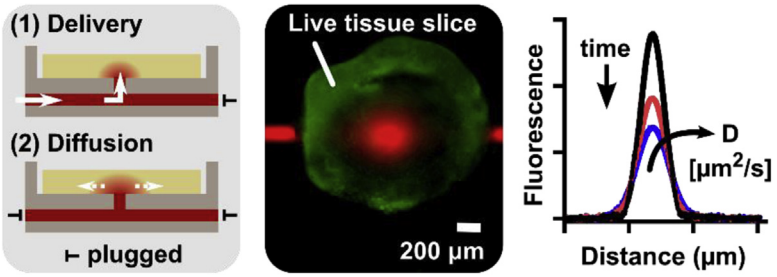
HIGHLIGHTS

- A novel method quantifies diffusion of bioactive analytes in live tissue.
- A microfluidic platform delivered fluorescent analytes noninvasively to tissue.
- Diffusion was monitored by time-lapse, widefield fluorescence microscopy.
- The method, Micro-IOI, was validated against theory and existing methods.
- Micro-IOI quantified the effective diffusion of cytokines in live lymph node tissue.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 22 October 2017
Received in revised form
15 November 2017
Accepted 17 November 2017
Available online xxx

Keywords:
Local delivery
Inflammation
Tumor necrosis factor alpha
Interleukin-2
Interferon gamma

ABSTRACT

Communication and drug efficacy in the immune system rely heavily on diffusion of proteins such as cytokines through the tissue matrix. Available methods to analyze diffusion in tissue require microinjection or saturating the tissue in protein, which may alter local transport properties due to damage or rapid cellular responses. Here, we developed a novel, user-friendly method – Microfluidic Integrated Optical Imaging (micro-IOI) – to quantify the effective diffusion coefficient of bioactive proteins in live tissue samples ex vivo. A microfluidic platform was used to deliver picograms of fluorescently labelled cytokines to microscale regions within slices of murine lymph node, and diffusion was monitored by widefield fluorescence microscopy. Micro-IOI was validated against theory and existing methods. Free diffusion coefficients were within 8% and 24% of Stokes-Einstein predictions for dextrans and cytokines, respectively. Furthermore, diffusion coefficients for dextrans and proteins in a model matrix were within 1.5-fold of reported results from fluorescence recovery after photobleaching (FRAP). We used micro-IOI to quantify the effective diffusion of three cytokines from different structural classes and two different expression systems – tumor necrosis factor alpha (TNF-α), interferon gamma (IFN-γ), and interleukin-2 (IL-2), from human and mouse – through live lymph node tissue. This is the first method to directly measure cytokine transport in live tissue slices, and in the future, it should promote a deeper understanding of the dynamics of cell-cell communication and enable targeted immunotherapy design.

1. Introduction

Diffusion of secreted proteins through the extracellular space is fundamental to cell-cell communication and organized tissue-level responses. Diffusion, coupled with binding events and interstitial

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https://doi.org/10.1016/j.aca.2017.11.048
0003-2670/

fluid flow, establishes concentration gradients that are essential to tissue morphogenesis, directional cell growth, chemotaxis within and between tissues, and drug delivery [1–7]. In tissue, unlike in a simple buffer solution, diffusion is hindered by the geometry of the extracellular space, by binding to cell-surface receptors or the extracellular matrix (ECM), and by non-specific charge-charge interactions [8,9]. The degree of hindrance, or tortuosity, for a given analyte is quantified by comparing the "effective diffusion" in tissue to the "free diffusion" in buffer [10]. Tortuosity in brain varies based on the health and region of the tissue [11–13], and for bioactive molecules, may vary also with the presence of binding sites in the ECM and on cell surfaces [9]. Quantifying effective diffusion and tortuosity in organs such as the brain has provided critical insights into the mechanisms of drug delivery and inflammatory diseases [10,14]. Unfortunately, localized diffusion data for bioactive proteins in smaller organs remains difficult to obtain. This paper describes a new analytical method to quantify diffusion of bioactive proteins in live tissue, specifically in lymph node slices, and provides the first diffusion coefficients for cytokines in healthy lymph node tissue.

Several techniques are available to analyze diffusion of macromolecules through biological tissues [10,15–17], but these may not be optimal for use with bioactive molecules in sensitive tissue samples. The gold-standard method is pressure ejection of fluorescently-labelled proteins coupled to integrated optical imaging (IOI), as pioneered by Nicholson [8,10,18]. Pressure ejection approximates a point source by delivering a small volume of analyte to the tissue through a micropipette as small as 1–2 μm at the tip [19,20]. Timelapse images are collected and analyzed by linescan to quantify the spread of the protein over time. This approach has been used productively for 25 years, predominately in brain, to quantify diffusion of fluorescently labelled probes and thus elucidate the geometric properties of the extracellular space. However, probe insertion causes mechanical damage to the tissue that generates rapid reactivity [21,22]. Diffusion distances for metabolites and proteins through tissue are on the order of 50–200 μm [6], therefore, danger signals could potentially extend within minutes of probe insertion over a region ~ 100–400 μm in diameter, the same length scale as substructures within the lymph node and other organs. Local damage surrounding the probe may be particularly detrimental to reactive organs such as the lymph node, whose rapid response to tissue damage [23] may alter the local microenvironment. An alternative method is fluorescence recovery after photobleaching (FRAP), in which the sample is saturated with a fluorescently-labelled protein and locally photobleached. Timelapse images are collected to quantify diffusion of the remaining protein into the bleached area over time [16]. FRAP has been used to quantify diffusion of protein-sized analytes in both gels [24,25] and tissue [26–28], using inert dextrans and proteins such as albumin. However, soaking lymph node tissue in bioactive cytokines may induce a rapid cellular response that could alter transport properties.

Methods are needed to analyze protein diffusion in the lymph node in order to inform the development of targeted immunotherapies and reveal mechanisms of cell-cell communication in adaptive immunity. The lymph node is highly structured, much like the brain, and its organized structure is critical for proper initiation of adaptive immunity [29–32]. Cell-cell communication in the lymph node is transmitted largely through secreted proteins called cytokines [33,34], which serve as drug targets because their local concentrations guide inflammatory and immune events [35–38]. Cytokines act both locally and across the node, indicative of a diffusing signal [39–41]. Many cytokines bind the extracellular matrix, which may contribute to gradient formation [42,43], and convection by interstitial flow likely also affects their distribution [44]. Despite intense interest in building predictive models of cytokine signaling for mechanistic studies and immunotherapy design [45–47], the diffusion coefficient of cytokines through lymph node tissue remains unmeasured.

In this paper, we describe a new method to quantify diffusion of bioactive proteins in live lymph node tissue. In order to limit local tissue damage and reactivity, we utilized microfluidic delivery of the protein in combination with IOI (Micro-IOI). A microfluidic channel terminating in a port was used to deliver bioactive cytokines to discrete regions of lymph node tissue. IOI image-analysis methods were developed to accommodate the background of the microfluidic channel, and the resulting Micro-IOI method was validated against FRAP in slices of agarose gel and validated for free diffusion of both dextrans and proteins. Next, the method was used to quantify the diffusion in tissue of three murine cytokines: IL-2, TNF-α, and IFN-γ, which play critical roles in the immune response and differ in molecular weight, oligomerization, and structure. We sought to isolate the effect of glycosylation on diffusion by testing cytokines expressed in mammalian cells versus in *E. coli* and finally calculated the tortuosity experienced by these proteins through the tissue.

2. Materials and methods

2.1. Preparation of fluorescently-labelled probes

Fluorescein-labelled dextran, anionic (FITC-dextran) in varying molecular weights and Alexa Fluor 647-labelled 10-kDa dextran were obtained from Life Technologies and stored at −20 °C in 1x phosphate buffered saline (PBS: 137 mM Sodium chloride, 10 mM sodium phosphate dibasic, 2.7 mM potassium chloride, and 1.8 mM potassium phosphate monobasic). Recombinant murine TNF-α, IFN-γ, and IL-2, expressed in *E. coli*, were obtained from Peprotech, and recombinant human TNF-α, IFN-γ, and IL-2, expressed in HEK 293 cells, were obtained from Acro Biosystems. Cytokines were covalently conjugated to a fluorophore by incubating with excess Alexa Fluor 647 NHS-ester (Life Technologies) for 1 h at room temperature, followed by 2 h at 4 °C. Excess dye was removed by repeated centrifugation through an EMD Millipore Amicon Ultra-2 centrifugation unit (Fisher Scientific) with a 3-kDa molecular weight cut-off. The degree of labelling was determined using a Nanodrop 1000 (Thermo Scientific), and was 3, 6, and 2 mol dye/mol protein for mouse IL-2, TNF-α, and IFN-γ, respectively. The degree of labelling for human IL-2, TNF-α, and IFN-γ, was 1.4, 2, and 2.6, respectively. Labelled cytokines were stored at −20 °C in 1x PBS.

2.2. Microfluidic device fabrication

The three-layer device was fabricated according to published procedures [48]. Briefly, the top and bottom layers (tissue culture chamber and microfluidic channels) were fabricated using standard soft lithography. Transparency masks were drawn in AutoCAD LT 2015 and printed at 20,000 DPI (CAD/Art Services Inc, Bandon OR). Master molds were fabricated using SU-8 3050 photoresist (Microchem, Westborough MA, USA) on 3″ silicon wafers in a class 1000 cleanroom. Polydimethylsiloxane (PDMS) was used at a 10:1 ratio of silicon elastomer base to curing agent (Ellsworth Adhesives, Germantown WI, USA) to create replicas of the device. Inlet and outlet holes were punched into the top layer prior to bonding using a 0.75 mm I.D. tissue punch (World Precision Instruments, Sarasota FL, USA) to accommodate nonshrinkable PTFE TT-30 tubing with 0.012″ I.D. and 0.009″ wall thickness (Weico Wire, Edgewood NY, USA) for delivery. The tissue culture chamber (top layer) was punched using a 12-mm tissue punch. The middle layer (exit port)

Please cite this article in press as: A.E. Ross, R.R. Pompano, Diffusion of cytokines in live lymph node tissue using microfluidic integrated optical imaging, Analytica Chimica Acta (2017), https://doi.org/10.1016/j.aca.2017.11.048