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Increasing insulin measurement throughput by fluorescence anisotropy imaging immunoassays

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Human Islet Research Network

Lili Liang

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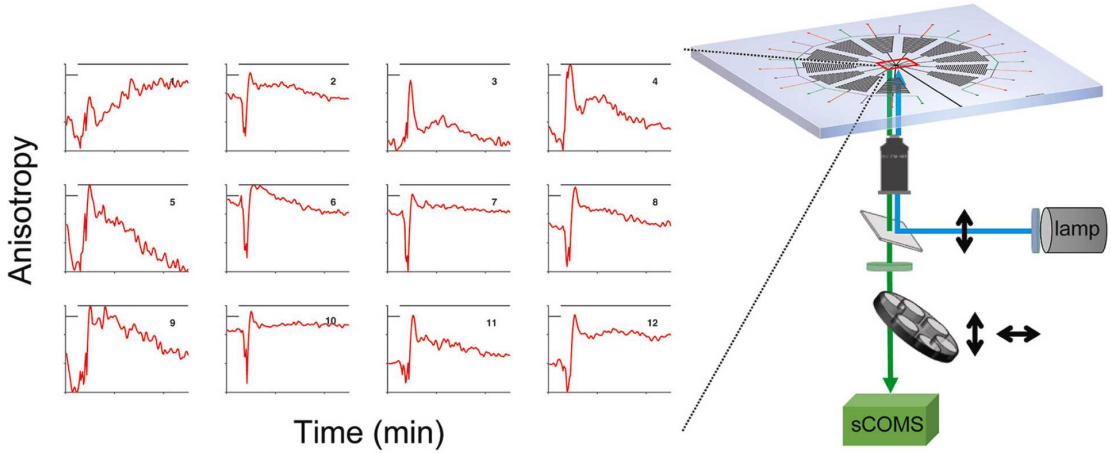
ABSTRACT

Insulin secreted from islets of Langerhans is the main hormone to reduce blood glucose. Examination of insulin secretion patterns at the single islet level reveals functional differences in the timings and patterns of release. This heterogeneous response highlights the importance of developing systems to measure dynamic release from small numbers of islets in parallel. Toward this, we describe fluorescence anisotropy imaging immunoassays as a relatively simple method for increased throughput of islet secretion measurements. In this system, vacuum pressure from a syringe pump pulled perfusate from 12 islet chambers and reagents into 12 parallel mixing channels for a competitive immunoassay. Light from a Xe arc lamp was filtered and polarized prior to focusing on the microfluidic device at the region where the 12 mixing channels converged. Emission was collected and passed through vertical and horizontal emission polarizers housed in an automated filter wheel before being imaged with a sCMOS camera for the determination of anisotropy. This microfluidic system was tested by monitoring insulin release from groups of murine and human islets. Heterogeneity was observed in the islet traces; however, the presence of islets affected the resistance of the islet chambers, hampering insulin quantification. Nonetheless, this microfluidic system is a step towards increasing the throughput of hormone release measurements from islets of Langerhan.

Graphical abstract



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GUIDELINES

Introduction

Insulin secreted from β-cells located in pancreatic islets of Langerhans is the main hormone to reduce blood glucose levels. Morphological (size, composition, and architecture) as well as functional differences (electrical activity, glucose responsiveness, and secretion rates) exist between the ~1,000,000 islets in a human pancreas [1–4]. These differences arise, in part, during the process of pancreas development when receiving distinct signals from surrounding tissues and microenvironment [5,6]. Heterogeneity can also occur during adaptation to physiological or pathological conditions, such as pregnancy, obesity, and diabetes [6–9]. However, the extent of heterogeneity and how islets respond in synchrony to metabolic challenges in the face of this heterogeneity is unknown. Because heterogeneous phenotypes are more likely to be revealed in small group sizes, it is important to develop a system that can measure the functional response of small numbers of islets in parallel to examine this behavior.

Insulin secretion measurements are typically performed using antibody-based assays, for example enzyme-linked immunosorbent assays. While these assays have high

sensitivity and low limits of detection, they require multiple wash steps and long assay times. In addition, they are challenging to perform in an online format for real-time results. Other types of immunoassays have been developed to reduce assay time and enable online detection [10–15]. For example, electrophoretic assays on microfluidic devices have been developed that enable insulin dynamics to be measured every ~10 s [12–17]. In these competitive immunoassays, insulin released from islets competes with fluorescently labeled insulin (insulin*) for a limited amount of anti-insulin antibody (Ab). The reaction produces both Ab-bound (B) and free (F) insulin*, with the B/F ratio inversely proportional to the unlabeled insulin concentration. The amounts of the fluorescent species are detected after a rapid electrophoretic separation. Due to the sensitivity of these assays, they have been used for examining single islet insulin secretion dynamics and have been modified to measure release from multiple single islets in parallel [16,17]. While highly sensitive and rapid, this electrophoresis-based method is difficult because of the shallow microchannels that are required for minimizing Joule heating, which leads to laborious chip fabrication processes and high chances of clogging. In addition, the number of islets that can be examined in parallel may be limited by the potential for arcing if the device architecture becomes too dense.

Fluorescence anisotropy (FA) or fluorescence polarization (more frequently used in earlier publications and clinical literature) is an all-optical method that can be used with immunoassays without the need to separate the bound and free species, providing significant advantages for rapid screening and high throughput assays [18–22]. FA measures the degree of depolarization in the emission light after excitation with linearly polarized light [20]. Fluorophores that are aligned with the excitation light become excited, and the depolarization of the emission is a function of the rotational diffusion of the fluorophores, which is inversely proportional to their molar volume. For the FA competitive insulin immunoassay, insulin* has a high rotational diffusion compared to the Ab-insulin* complex, resulting in a smaller anisotropy value. The total anisotropy of the solution is the sum of the anisotropy of each species weighted by their fractional amounts. Therefore, for every insulin concentration, a unique B/F value and a corresponding total anisotropy is produced. To measure anisotropy, the solution is excited with linearly polarized light and the emission parallel and perpendicular with respect to the direction of excitation polarization is determined. This total anisotropy (r) is calculated as: **Equation 1**

$$r = \frac{I_{II} - G * I_{\perp}}{I_{II} + 2{*}G * I_{\perp}}$$

where I_{II} and $I_{\perp}$ are the fluorescence intensities measured parallel and perpendicular to the direction of the polarized excitation, and G is the ratio of detection sensitivity for the two polarization directions. FA immunoassays have been implemented for measurements of insulin from both single and groups of islets [23–26]; however, a single point measurement scheme was used, limiting the analysis to a single detection channel. To increase throughput, fluorescence anisotropy imaging (FAI) could be used to examine multiple channels simultaneously [22, 27]. FAI relies on the same principle as the single point measurements where polarized excitation is used

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while the individual polarized emission components are imaged either sequentially [21] or simultaneously [28,29].

We report a FAI system capable of monitoring insulin release from small numbers of islets in parallel. Groups of 12 competitive immunoassay systems were integrated into a single microfluidic system and FAI used to determine the B/F ratio as a function of time for each group. To simplify the device, the fluidics were controlled by a single syringe pump withdrawing liquid from all channels. Detection was achieved by imaging the horizontally and vertically polarized emission sequentially using an automated filter wheel and a sCMOS camera. This method was applied to monitor insulin release dynamics from 12 groups of murine and human islets in parallel. The method was shown to be robust and sensitive, but some caveats were observed due to the withdrawal-based fluid system. Nevertheless, the method is a step towards a simple, high throughput system capable of measuring islet functional heterogeneity.

CRediT authorship contribution statement

Yao Wang: Investigation, Software, Writing – original draft, Writing - review & editing.
Damilola I. Adeoye: Investigation.
Yue J. Wang: Investigation, Resources.
Michael G. Roper: Investigation, Resources, Funding acquisition, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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MATERIALS TEXT

Chemicals and reagents

Sodium chloride **Emdmillipore Catalog #SX0420-1**

Calcium chloride **Emdmillipore Catalog #102391**

Sodium hydroxide **Emdmillipore Catalog #SX0590**

ethylenediaminetetraacetic acid (EDTA) **Emdmillipore Catalog #4010-OP**

Tween-20 **Emdmillipore Catalog #817072**

bovine serum albumin (BSA) **Emdmillipore Catalog #160069**

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Dextrose **Thermo Fisher Scientific Catalog #D16-1**

RPMI 1640 **Thermo Fisher Scientific Catalog #MT10040CV**

Gentamicin **Thermo Fisher Scientific Catalog #15710064**

Fetal Bovine Serum **Thermo Fisher Scientific Catalog #16140071**

Collagenase P from Clostridium histolyticum **Roche Diagnostics Catalog #11215809103**

Monoclonal insulin antibody **Meridian Life Science Catalog #E86211M**

Fluorescein isothiocyanate labeled insulin **Sigma Aldrich Catalog #I3661**

Nylon Syringe Filters
0.2 µm

Acrodisc, Pall Corporation AP-4436

Experimental

1 Chemicals and reagents

Sodium chloride, calcium chloride, sodium hydroxide, ethylenediaminetetraacetic acid (EDTA), Tween-20, and bovine serum albumin (BSA) were from EMD Chemicals (San Diego, CA). Dextrose, RPMI 1640, gentamicin, and fetal bovine serum were from Thermo Fisher Scientific (Waltham, MA). Collagenase P (from Clostrdium histolyticum) was acquired from Roche Diagnostics (Indianapolis, IN). Monoclonal insulin antibody (Ab) was purchased from Meridian Life Science, Inc. (Saco, ME). Fluorescein isothiocyanate labeled insulin (insulin*) and other reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless noted otherwise. All solutions were made with Milli-Q (Millipore, Bedford, MA) 18 MΩ cm ultrapure water and filtered using 0.2 µm nylon syringe filters (Pall Corporation, Port Washington, NY).

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Immunoassay reagents (insulin* and Ab) were prepared in TEAT-40 (pH **7.4**) composed of 25 mM tricine, 40 mM NaCl, 1 mM EDTA, 0.1% Tween-20 (w/v), and 1 mg mL^{-1} BSA. 200 nM of the insulin* and Ab were placed in their respective reservoirs for experiments. Islets or insulin standards were placed in a balanced salt solution (BSS) (pH **7.4**) that consisted of 125 mM NaCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 5.9 mM KCl, 25 mM tricine, 1 mg mL^{-1} BSA, and the appropriate glucose concentration as described in the text.

2 Microfluidic device and system

The microfluidic device was fabricated in borosilicate glass (Telic Company, Santa Clarita, CA) using wet chemical etching techniques previously described [15,26]. Microfluidic channels were 50 µm deep and 75 µm wide in the middle measured by an SJ-410 surface profiler (Mitutoyo Corp., Aurora, IL). Fluidic access holes for immunoassay reagents and islet chambers were drilled using 0.02" and 0.012" diamond-tipped drill bits (Industrial Power Tool and Abrasives, NY), respectively. Fluidic reservoirs (IDEX Health and Science, Oak Harbor, WA) were bonded to the microfluidic device according to the manufacturer's instructions. A syringe pump (Harvard Apparatus, Holliston, MA) was connected to the common outlet on the device with Tygon tubing (0.02" ID × 0.06" OD, Cole-Parmer North America, Vernon Hills, IL) through a fingertight fitting (IDEX Health and Science).

3 Optical detection system

The microfluidic device was placed on a motorized XY microscope stage (Zaber Technologies Inc., Vancouver, British Columbia, Canada) mounted on a Nikon Eclipse Ti–S inverted microscope (Nikon Instruments Inc., Melville, NY). Excitation light from a Xenon arc lamp (Lambda XL, Sutter Instruments, Novato, CA) was sent through a 485 ± 35 nm bandpass filter (Semrock, Rochester, NY) and coupled to the microscope with a liquid light guide. The light then passed a linear polarizer (WP25M-VIS, Thorlabs Inc., Newton, NJ) and was reflected by a dichroic mirror (FF506-Di02-25 × 36, Semrock) and focused onto the microfluidic device using a 10 × 0.5 NA objective (Nikon). Fluorescence emission was collected by the same objective and filtered using a 536 ± 40 nm bandpass (Semrock) emission filter. The emission was sent through an automated emission filter wheel (Applied Scientific Instrumentation, Eugene, OR) that contained linear polarizers, oriented parallel and perpendicular with respect to the excitation polarizer, and imaged with a sCMOS camera (Prime BSI express, Photometrics, Tucson, AZ). Each image was acquired with a 10 s exposure time. Micro-manager software [30,31] was used to control image capture, filter wheel, and the XY microscope stage movement. A microscope environmental chamber (World Precision Instruments, Sarasota, FL) was used to maintain the device and solutions for all experiments at 🌡 **37 °C** unless otherwise noted.

4 Isolation and culture islets of Langerhans

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Murine pancreatic islets were isolated from CD-1 male mice (Charles River Laboratories, Wilmington, MA) according to the Florida State University Animal Care and Use Committee (Protocol number 202000078) as previously described [32]. Isolated islets were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U mL^{-1}penicillin, 100 µg mL^{-1}streptomycin, and 10 µg mL^{-1} gentamycin (Eppendorf North America, Enfield, CT) at **37 °C** and 5% CO_2. Islets were used within 4 days after isolation.

Human islets, obtained from Prodo Laboratories (Aliso Viejo, CA), were from deidentified cadaveric organ donors and, therefore, exempt from Institutional Review Board approval. The donor characteristics are given in **Table S1**. Human islets were cultured at **37 °C** and 5% CO_2 in PIM(S) islet-specific media (Prodo Laboratories).

Prior to each experiment, islets were washed in a dish of prewarmed BSS containing 3 mM glucose for 10 min. Groups of five murine islets or seven human islets were then loaded into each of the 12 islet chambers on the microfluidic device.

	Donor 1 (HP-21247-01)	Donor 2 (HP-21272-01)
Sex	M	M
Age	53	39
Height (in)	72	71
Weights (lbs)	230	208
BMI	31	29
HbA1c (%)	5.9	5.4

Table S1. Characteristics of human islet donors.

5 Data analysis

The average fluorescence intensity from each parallel and perpendicular image pair was measured from a region of interest (ROI) in ImageJ [33] and converted to anisotropy using **Equation (1)**. Each point on the calibration curves is the average anisotropy from 5 consecutive measurements with error bars showing ±1 standard deviation (SD). Calibrations plots were fitted with a four-parameter logistic function using a MATLAB (MathWorks, Natick, MA) script written in-house. The limit of detection (LOD) was taken as the concentration of insulin that decreased the anisotropy value of the blank solution by 3 times the SD. For presentation, islet traces were smoothed using the built-in smoothing 'rloess' function in MATLAB with a span of 7 points, which can be called by smooth(y, 'rloess'). This smoothing

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function fits a weighted least squares 2nd degree polynomial to the span of data.

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