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REVERSIBLY-SEALED MULTILAYER MICROFLUIDIC DEVICE FOR INTEGRATED CELL PERFUSION AND ON-LINE CHEMICAL ANALYSIS OF CULTURED ADIPOCYTE SECRETIONS

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Abstract

A three-layer microfluidic device was developed that combined perfusion of cultured cells with on-line chemical analysis for near real-time monitoring of cellular secretions. Two layers were reversibly sealed to form a cell chamber that could allow cells grown on coverslips to be loaded directly into the chip. The outlet of the chamber was in fluidic contact with a third layer that was permanently bonded. Perfusate from the cell chamber flowed into this third layer where a fluorescence enzyme assay for non-esterified fatty acid (NEFA) was performed on-line. The device was used to monitor efflux of NEFAs from cultured adipocytes with 83 s temporal resolution. Perfusion of murine 3T3-L1 cultured adipocytes resulted in an average basal concentration of $24.2 \pm 2.4 \mu\text{M}$ NEFA (SEM, $n = 6$) detected in the effluent corresponding to $3.31 \times 10^{-5} \text{ nmol cell}^{-1} \text{ min}^{-1}$. Upon pharmacological treatment with a β -adrenergic agonist to stimulate lipolysis, a 6.9 ± 0.7 -fold (SEM, $n = 6$) sustained increase in NEFA secretion was observed. This multilayer device was capable of monitoring NEFA from ~6 250 adipocytes. This multilayer device provides a versatile platform that could be adapted for use with other cell types to study corresponding cellular secretions.

Keywords

Adipocytes; microfluidics; enzyme assay; integration

Introduction

Discoveries over the past couple decades have shifted the perception of adipocytes (fat cells) from metabolically inert cells that only store excess energy to active cells that secrete numerous moieties, have endocrine function, and play an important role in diseases such as type 2 diabetes mellitus, hypertension, and cardiovascular disease.[1] Better understanding of adipocytes requires resolving outstanding questions regarding adipogenesis, adipocyte metabolism, and inflammation.[2] For instance, questions to be resolved include how Wnt ligands affect mature adipocyte metabolism and what signaling pathways impact the secretion of non-esterified fatty acids (NEFAs). A convenient method to monitor secretion

of NEFAs from adipocytes would be useful to aid in these studies because such measurements can be directly related to adipocyte metabolism and function. Most studies of NEFA secretion from adipocytes are performed using static incubation with periodic sampling, but these methods neglect kinetic information and limit the types of experiments that can be done. For kinetic studies, perfusion systems comprised of a packed column of cells are used with manual collection of effluent;[3–7] however, these systems are reagent- and labor-intensive.

Microfluidic systems may offer a convenient alternative to these macro-scale approaches. Microfluidic devices are a useful platform for studying cell physiology because a controlled cellular environment can be integrated with powerful analytical methods in a miniaturized system.[8] For perfusion and secretion measurements, using microfluidics can save time and reagent relative to traditional methods involving fraction collection and off-line analysis.[9–15] Examples of approaches that have been developed include electrophoretic immunoassays to study insulin secretion from pancreatic islets,[9, 10] amperometry to monitor lactate produced from cardiomyocytes,[12] and enzyme assays to measure nitric oxide release from peritoneal macrophages.[15]

Adipocytes have several intrinsic properties, which make their incorporation on-chip difficult including lengthy culture times (~2 weeks) to differentiate adipocyte cell lines, fragility of lipid-laden adipocytes, high buoyancy, and secretion of hydrophobic moieties. Previously, we have developed a microfluidic platform to study adipocytes that addressed many of these difficulties.[13] Device modifications included growth of cells on coverslips enabling loading of cells the time of the experiment to circumvent lengthy culture times, location of inlet and exit outlets to minimize shear stress, incorporation of an in-line weir to confine non-adherent adipocytes to the cell chamber, and fabrication in glass to prevent adsorption of secreted hydrophobic moieties. This dual-chip system was comprised of two separate glass chips (a cell perfusion chip and an on-line enzyme assay chip), which monitored glycerol release from adipocytes. The system used in our previous study advanced the state-of-the-art in adipocyte monitoring; however, it contained many external capillary connections that increased reagent waste and the time to transfer fluids between chips.

In this study, we present an improved microfluidic system for monitoring cellular secretions from cultured adipocytes. A reversibly-sealed three-layer chip was fabricated to combine the cell chamber and the enzyme assay network into a single working device that lessens the amount of external connections required. This three-layer device had a smaller cell chamber that sampled 8-fold fewer fat cells than the previous design and decreased reagent consumption by an order of magnitude. Moreover, we furthered our work using an adipocyte cell line (differentiated murine 3T3-L1 cells) by extending the current device to monitor fatty acid release rather than glycerol efflux from adipocytes. Fatty acids and glycerol are both released from triglycerides stored within adipocytes via lipolysis. Measuring NEFAs and glycerol separately is important because they are not always secreted in the 3:1 ratio expected from triglyceride structure (i.e., 3 fatty acid moieties for every glycerol in a triglyceride). Extension of the method to NEFA release also is of interest because fatty acids have more established roles in disease such as degradation of vasculature structure[16, 17] and insulin secretion.[18] The results further show that use of an enzyme assay for detection generates a system for enabled continuous read-out of cellular secretions that is versatile enough to be adapted to monitor different secreted moieties.

Experimental Section

Chemicals and Reagents

The non-esterified fatty acid kit (NEFA-HR(2) Series) for colorimetric detection was obtained from Wako Chemical USA Inc. (Baltimore, MD). Amplex UltraRed was purchased from Invitrogen (Carlsbad, CA). Isoproterenol hydrochloride was obtained from Tocris Bioscience (Ellisville, MO). Fatty acid-free bovine serum albumin (BSA), dimethyl sulfoxide (DMSO), cell culture reagents, and Hanks balanced salt solution (HBSS) were purchased from Sigma (St. Louis, MO). NEFA standards and the glucose-BSA-DMSO solution perfused over the cells were made using HBSS as solvent. Other solutions were made using Milli-Q (Millipore, Bedford, MA) 18 M Ω · cm deionized water. Solutions flowed through the microfluidic device were filtered using 0.2 μ m nylon syringe filters (Fisher).

Fabrication of Microfluidic Chip

The multilayer microfluidic device (Figure 1a) was fabricated using wet-chemical etching techniques described previously.[13] The multilayer device consisted of three etched glass wafers: a bottom wafer for the enzyme assay, a middle wafer for the fluidic transfer channel and the bottom portion of the cell chamber, and a top wafer for the fluidic network and the top portion of the cell chamber. The top wafer was reversibly sealed to the bonded bottom two wafers. The enzyme assay channels were etched to a depth of 60 μ m using a hydrofluoric acid solution, the bottom portion of the cell chamber was 450 μ m deep, and the top wafer had 5 μ m inlet and outlet channels to serve as a weir and both a 100 μ m deep moat and top portion of the cell chamber (Figure 1b). The cell chamber was 1.25 cm long $\times$ 0.2 cm wide. Transfer channels were drilled using 360 μ m drill bits (Kyocera, Irvine, CA) for side and between-layer holes and 1 mm drill bits (Euro Tool, Inc., Grandview, MO) for the inlet and outlet holes in the top layer. The bottom wafer and middle wafer were bonded together with the perfusate channel aligned with the fluidic transfer channel. Two perfusion reservoirs (Upchurch N-124H, Oak Harbor, WA) were attached to the top wafer for fluidic connection. Fused silica capillary (Polymicro, Phoenix, AZ) was glued into the side of the chip at drilled inlet and exit channels using epoxy (EPOXY 5, Northeast Hobby Products Co., Chelmsford, MA) and allowed to cure for 1 hour prior to use.

Adipocyte Culture

Murine 3T3-L1 preadipocytes were cultured using a method previously described.[13] Briefly, preadipocytes were cultured in 10 cm Petri dishes at 37 °C and 10% CO₂ until confluent. Preadipocyte media consisted of Dulbecco's modified Eagle medium with 10% calf serum. Two days post-confluence, fetal bovine serum, insulin, dexamethasone, and isobutylmethylxanthine were added to the media to induce differentiation.[19] Every 2 days, the media was replaced. Cell experiments were performed using adipocytes 13 days after differentiation was induced. For ease of transfer from culture dish to chip, cells were grown on glass coverslips (2 $\times$ 12.5 $\times$ 0.15 mm³, Sigma) that were cut using a diamond-tipped scorer to fit the cell chamber in the microfluidic device. The coverslips were sterilized with 70% ethanol and placed in the Petri dishes prior to plating cells.

Multilayer Chip Reversible Sealing and Cell Perfusion

Reversible sealing of the top wafer to the bonded bottom two wafers was obtained by applying high vacuum grease (Fisher) and a pre-cut poly(tetrafluoroethylene) (PTFE) sheet (125 μ m thick, Small Parts, Inc., Miramar, FL) to the 100 μ m deep moat before pressing the two components together. Sufficient contact between the bonded portion of the device and the top wafer was ensured by use of an in-house built compression frame comprised of two

pieces of aluminum ($16.5 \times 10 \times 0.5 \text{ cm}^3$) screwed together (Figure 1c). An acrylic top cover was used in place of the aluminum top cover in some experiments to observe flow of dye through the chip. Confinement of fluid flow to the cell chamber, washout of the chamber, transfer of fluid to the enzyme assay portion of the device, and appropriate flow split ratio were verified by flowing solutions containing dye through the chip.

For secretion measurements, coverslips supporting ~6 250 differentiated adipocytes were transferred to the cell chamber. The cells were perfused at $8 \mu\text{L min}^{-1}$ with HBSS containing 5 mM glucose, 2% (v/v) DMSO, and 2% (mg/mL) fatty acid-free BSA in the absence or presence of 20 μM isoproterenol. The fatty acid-free BSA was added to the perfusate buffer to prevent surface adsorption and to maintain adipocyte viability. The addition of BSA did not alter the enzyme assay kinetics or the fluorescence signal detected. DMSO was found to be helpful in maintaining stable fluid flow over the cells. Without the addition of 2% DMSO, the buffer flowed around the cells and did not sample the cellular release. Throughout the secretion experiments, the cells remained adherent to the glass coverslips.

The perfusate solution was prewarmed to 37 °C prior to introduction onto the chip. Moreover, the cell chamber was maintained at 37 °C as measured by a digital thermometer (Fisher) by placing a thin-film resistive heater (Minco Products, Inc., Fridley, MN) on top of the compression frame over the cell chamber area. Fluid flowed into the device after passing through a 6-port valve (Valco Instruments Co., Inc., Houston, TX) used for stream selection and fused silica capillary (28 cm, 250 μm i.d.) connected to a syringe pump (model 402, CMA Microdialysis, North Chelmsford, MA) fitted with 1 mL syringes (Hamilton, Reno, NV) (Figure 2a).

Enzymatic Assay and On-line Mixing

A fluorescence-based enzyme assay for NEFAs was developed by adding the hydrogen peroxide-sensitive dye Amplex UltraRed to a commercially-available absorbance-based enzyme assay for NEFAs (Wako Diagnostics) as depicted in Figure 4a. The Wako Reagent A was reconstituted using Solvent A from the NEFA-HR(2) Series kit according to the manufacturer's instructions; however, Reagent B was reconstituted with water instead of Solvent B and, thus, removed 3-methyl-N-ethyl-N-(β -hydroxyethyl)-aniline (MEHA) which normally would form a colored, oxidative product for absorbance-based detection. Amplex UltraRed was reconstituted with DMSO to result in a 925 μM solution after on-chip mixing. The dye, sample, and enzyme reagents were mixed on-line and allowed to react for 5 min prior to detection on-chip.

The assay was performed by flowing sample (either NEFA standards or cell perfusate) through the cell chamber and to the enzyme assay portion of the device. Out of the $8 \mu\text{L min}^{-1}$ flow through the cell chamber, 3% of the flow or 250 nL min^{-1} was diverted through the transfer channel into the enzyme assay channel. The desired flow split was obtained by plumbing a piece of fused silica capillary (6.5 cm, 150 μm i.d.) at the exit reservoir. Upon entering the enzyme assay portion of the device, the perfusate flow was mixed in a 1:1:1 ratio with Reagent A and a premixed solution of 2 parts of Reagent B (minus MEHA) and 1 part of Amplex UltraRed. Offline tests of the premixed solution showed minimal change in the fluorescence of the solution (i.e. the dye remained unreacted) over several hours if the solution remained out of the light. Online flow rates for Reagent A and the premixed solution of Reagent B and Amplex UltraRed were 250 nL min^{-1} each by use of syringe pump. The overall flow rate through the enzyme assay mixing and incubation channel was 750 nL min^{-1} . The enzyme assay channel dimensions[13] resulted in a 5 min mixing and incubation time prior to detection. The 5 min incubation time was determined in off-line experiments to generate sufficient sensitivity.

LIF Detection

A Zeiss Axiovert 100 inverted microscope with epifluorescence optics was used to monitor the on-line enzyme assay via fluorescence detection. The 543.4 nm line of a He-Ne laser (Melles Griot, Albuquerque, NM) was directed through a 40x, 0.6 numerical aperture, long-working distance objective (Carl Zeiss, Inc., Thornwood, NY) for detection. Fluorescence was detected by a photon counting detector (Thermo Oriel, Stratford, CT) at 2 Hz using a LabVIEW program written in-house after passing through an appropriate filter set (Omega Optical, Inc., Brattleboro, VT) and a 1 mm pinhole spatial filter. For data analysis and graphing, Microsoft Excel 2000 (Microsoft, Redmond, WA), Prism 3.03 (GraphPad Software, Inc., La Jolla, CA), and Cutter 5.0[20] were used. A Canon EOS Digital Rebel XT camera (Cannon U.S.A. Inc., Lake Success, NY) was used to acquire visual images.

Computational Modeling of the Cell Perfusion Chamber

The fluid flow dynamics of the cell chamber region of the multilayer chip was modeled using COMSOL Multiphysics 3.5a (Comsol, Inc., Burlington, MA). The flow was defined to be laminar with a volumetric flow rate of $8 \mu\text{L min}^{-1}$ through the cell chamber. Using the Stokes-Einstein relationship to adjust to 37°C , the diffusion coefficient used for oleic acid was $5.05 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$. [21] The density of water was 993 kg m^{-3} and the viscosity of water (37°C) was $6.90 \times 10^{-4} \text{ Pa s}$. The cell chamber volume used was $1.25 \text{ cm long} \times 0.2 \text{ cm wide} \times 0.04 \text{ cm deep}$ (0.01 cm from the top wafer and 0.03 from the middle wafer after accounting for the presence of the 0.015 cm thick coverslip). Cell flux values accounted for dilution based on the cell chamber volume and flow rate. The cells were modeled to release oleic acid. Release from the cells was modeled to occur $31.25 \mu\text{m}$ above the coverslip, which was equivalent to the radius of an average-sized differentiated adipocyte.

Results and Discussion

Fluid Dynamics of Multilayer Chip

The three-layer integrated device contained a cell perfusion chamber between the top two reversibly-sealed wafers and a fluidic network for on-line mixing and detection of an enzymatic reaction between the bottom two bonded wafers (Figure 1). Fluid was transferred between the wafers via a hole drilled through the middle wafer. Cells could be grown on coverslips and transferred to the reversibly-sealed cell chamber enabling facile loading of cells and reuse of the chip. This approach also allowed adipocytes to be differentiated prior to placing in the chip. Effluent from the perfusion portion of the device flowed into the enzyme assay portion of the device for monitoring of cellular secretions.

An important performance consideration for the chip is dynamic response. A time delay between chemical secretion and detection is inevitable due to the time to flow in reagents and to allow for on-line reaction. Along with the time delay, a temporal distortion of the kinetics of secretion will occur due to flow and diffusional broadening that occurs during mass transfer from the cell chamber to the detection point. This distortion may be compounded by similar dispersion of any chemicals added from the perfusion line to initiate secretion.

To characterize the dynamics of the system, solution perfused through the chip was switched between $30 \mu\text{M}$ resorufin and blank (water) using a stream selector valve while monitoring the change in fluorescence at different points downstream in the chip (see Figure 2). For these experiments, flow rates mimicked those used for cell experiments i.e., solutions were pumped through the device at $8 \mu\text{L min}^{-1}$, reduced to 250 nL min^{-1} by a flow split at the sampling point, then increased to 750 nL min^{-1} in the enzyme assay portion by addition of water flow in the reagent channels. As expected, the temporal distortion increased the farther

downstream the measurement was taken. According to these results, the distance between the mixing point and the capillary inlet, which encompasses the cell chamber and the flow split, contributed the most to the overall distortion. The temporal resolution of the entire system, defined as 10 – 90% rise time at the detection point after a step change at the valve, was 83 ± 3 s (SD, $n = 5$), which included the time to switch solutions. The time delay between switch and detection was 9.1 ± 0.6 min (SD, $n = 5$).

Characterization of Cellular Release with Finite Element Model

Temporal response only could be experimentally assessed by making changes before the cell chamber. To better understand the dynamics for measurements of efflux from cells within the cell chamber, we modeled a step change in flux from cells in the bottom of the chamber as shown in Figure 3. A step increase of ~6x in secretion, such as might follow a pharmacological treatment, resulted in a 10 – 90% rise time of 56 s and was diluted by ~1.3-fold due to flow of perfusion buffer for the case of $80 \mu\text{L min}^{-1}$ flow rate and $5.05 \times 10^{-11} \text{ m}^2 \text{ s}^{-1}$ diffusion coefficient. The temporal resolution for the change in cellular release from basal to treated levels at the outlet channel of the chamber provided a measure of the minimum amount of time it would take to detect a change in cellular secretion after pharmacological treatment. This time did not account for broadening of the chemical front as fluid flowed downstream.

The model also depicted interaction between adipocytes as fluid flowed across the chamber. Secretion from cells on the left passed over downstream cells as the fluid flowed toward the right of the chamber. This adipocyte-adipocyte interaction could be interesting in future experiments if cells are perfused in tandem or time-dependent experiments are performed as well as for co-culture experiments using different cell types.

On-Line Enzyme Assay Characterization

Although a NEFA enzyme assay was commercially available and has been used previously for various applications including monitoring adipocyte metabolism *in vitro*[4–7, 22] and *in vivo* (in plasma),[23, 24] the enzyme assay was designed for absorbance-based detection. Conversion of the NEFA assay to fluorescence detection was desirable for on-chip monitoring and automation to improve sensitivity in microfluidic channels. The absorbance-based assay was converted to a fluorescence-based assay by the inclusion of Amplex UltraRed, a hydrogen peroxide-sensitive dye (Figure 4a).

Results from on-line calibration of the enzyme assay using step changes in fatty acid standards from 0 to 250 μM are illustrated in Figure 4b. The assay resulted in a linear response from the blank to at least 250 μM fatty acid (Figure 4c). Efficient mixing of reagents and a stable reaction was indicated by the 1 – 5% relative standard deviations (RSD) of the signals generated. The limit of detection (LOD) of the on-line fluorescence-based assay, as defined by the concentration that gave a signal 3 times the standard deviation of the blank, was 5 μM .

Maintaining the correct flow split was essential to maintaining the sensitivity of the assay. Verification of the proper flow split ratio was observed by flowing water containing food dye through the cell chamber and past the flow split into the enzyme assay portion of the device while flowing blank solutions through the side inlets. Ensuring sufficient contact between the reversibly-sealed layers as well as purging the system of air bubbles improved the reproducibility.

Non-Esterified Fatty Acid Secretion from Adipocytes

The multilayer device was used to measure adipocyte secretion dynamics by monitoring fatty acid release upon perfusion with the β -adrenergic receptor isoproterenol. Isoproterenol is known to stimulate lipolysis and the release of NEFAs by activating protein kinase A.[25] Basal measurements over ~20 min averaged $24.2 \pm 2.4 \mu\text{M}$ (SEM, $n = 6$) which corresponds to $3.31 \times 10^{-5} \text{ nmol cell}^{-1} \text{ min}^{-1}$. Upon switching to perfusion buffer containing $20 \mu\text{M}$ isoproterenol, a rise in NEFA concentration was observed that was 6.9 ± 0.7 -fold (SEM, $n = 6$) over basal measurements (see Figure 5a,b for individual traces and 5c for average). The rise in fatty acid levels upon treatment occurred within 2.5 min of exposure to isoproterenol, and the increase in release was sustained during treatment. A similar trend of a rapid, sustained increase in NEFA release upon isoproterenol treatment was observed previously using fraction collection and offline analysis of $\sim 2 \times 10^6$ primary rat adipocytes.[7] Interestingly, this result is in contrast to a more complex dynamic observed with glycerol as we previously reported.[13]

As a control to ensure that the fluorescence signal detected was not due to artifacts, enzyme reagents were replaced with buffer at the beginning and end of a cellular secretion experiment (Figure 5d). The signals observed without enzyme reagent were below the LOD of the assay and were similar to the unreacted dye alone. The characteristic NEFA release profile with a sustained increase in NEFA concentration upon treatment with isoproterenol was observed in the middle portion of the experiment showing that the cells were viable and secreting fatty acids during the experiment. The minimal signal detected without enzyme reagents present verified that enzymatic conversion of fatty acids was needed for the observed increases in fluorescence. Moreover, the experiment conveyed that Amplex UltraRed was not directly oxidized by cellular secretions or additives such as isoproterenol.

Comparison to Previous Work

In a previous study, we described a system comprised of two separate chips, one for cell perfusion and one for an on-line enzyme assay, capable of monitoring glycerol release from cultured adipocytes. The multilayer chip reported here has several advantages over the previous dual-chip system. Integrating the device eliminated the need for capillary to connect the two main portions of the device and reduced the transfer time by 64% or 0.84 min. Moreover, the multilayer device had a smaller cell chamber volume, which was flushed at a lower flow rate resulting in 10-fold decreased reagent consumption relative to the dual-chip system. Use of less perfusion buffer, which could contain pharmacological reagents such as isoproterenol, could lead to reduction in cost. (The enzyme reagent consumption was unchanged from previous work because the same flow rate was used for the enzyme assay.) The smaller cell chamber also reduced the number of cells required 8-fold from ~50 000 to ~6 250. The decrease in cells sampled enabled more coverslips from the same Petri dish to be tested, which may minimize biological variability. It also could allow complementary experiments using cells from the same dish to examine cellular content such as protein or mRNA levels. Furthermore, a decrease in the sample requirement could be beneficial for testing if the amount of cells was limited as is the case when using primary adipocytes. Finally, the new system was designed to measure NEFA rather than glycerol release. Abnormalities in NEFA secretion have established biological effects in a variety of disease state, and the ability to measure NEFA release would provide insight into adipocyte metabolism and function. The ease with which the system can be coupled with different enzyme assays suggests it could have wide use for studying a variety of cell types.

Conclusion

We developed a multilayer, integrated device that consisted of a perfusion cell chamber and a fluidic network for enzymatic reaction to enable monitoring of fatty acid release from cultured adipocytes in near real-time. The microfluidic device detected changes in fatty acid release from ~6 250 adipocytes with on-line mixing and detection of a fluorescence-based enzyme assay under basal conditions and with pharmacological treatment of isoproterenol and had a LOD of 5 μ M. The multilayer device utilized a reversible seal between the top two wafers to aid in transfer of cells grown on coverslips and reuse of the device. In the future, the device may be used for complementary glycerol secretion measurements, studies of genetically modified adipocytes to determine effects on lipolysis, or lipolysis measurements of primary adipocytes. Moreover, the device format could be extended for use with other cell types and corresponding enzyme assays to study cellular secretions.

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Fig. 1.

Chip Design (a) The multilayer device was comprised of three separately etched glass wafers that integrated a cell perfusion chamber and fluidic channels for on-line mixing of the fluorescence-based enzyme assay. (b) A side view of the cell chamber depicts the bonded and reversibly-sealed portions of the device. (c) An in-house built aluminum compression frame functioned to compress the three glass wafers together enabling reversible sealing as well as to serve as a microscope stage holder.

Fig. 2. Characterization of Fluid Dynamics of Multilayer Device (a) A diagram of the characterization experiment and the three detection points are shown. The change in fluorescence resulting from a step change was observed along the flow path of the chip at the end of the inlet capillary to the chip (point 1), prior to the mixing point of the enzyme assay (point 2), and at the detection spot (point 3). (b) Plot of the normalized resorufin step changes at different detection points. Rise times shown were calculated from the plots.

Fig. 3. Fluidic Modeling of NEFA Flux (a) Side view image of channel showing predicted NEFA concentration distribution within cell chamber at different times following a 6-fold step increase in NEFA efflux. (b) Simulations of the 6-fold step change. Model predicts a 56 s 10 – 90% rise time following step change. Arrow indicates time when the step change started ($t = 500$ s).

Fig. 4. On-Line NEFA Enzyme Assay Reaction and Calibration (a) The scheme of the fluorescence-based enzyme assay for NEFAs used for on-line mixing and detection is shown. Acyl-CoA synthetase (ACS), acyl-CoA oxidase (ACOD), peroxidase (HRP), and Amplex UltraRed were mixed on-chip with NEFAs (either from standards or effluent) to form a fluorescent product similar to resorufin. The R group represents a fatty acid tail of 3 to 17 carbon lengths (6 to 20 total carbons in length). (b) An example step-change calibration using the multilayer device is depicted. NEFA standards were flowed through the device ranging between 0 μM – 250 μM . (c) Calibration curve generated from the data in Figure 4a.

Fig. 5. On-line NEFA Secretion Monitoring from Cultured Adipocytes (a and b) Representative traces of NEFA release from differentiated adipocytes and response from treatment with 20 μM isoproterenol (indicated by the bar) are shown. (c) A plot of the averaged normalized release depicts a 6.9 ± 0.7 -fold (SEM, $n = 6$) increase in release of NEFAs upon isoproterenol treatment. The grey lines above and below the average response are $\pm$ SEM. (d) A control experiment was performed that monitored NEFA release in the presence and absence of enzyme reagents. Basal and isoproterenol-treated NEFA values were obtained once the enzyme reagents were added back to the system. The insert depicts the values detected during basal and isoproterenol treatment without enzyme reagents present.