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Development of a mix-and-read assay for human asprosin using antibody–oligonucleotide probes and thermofluorimetric analysis†

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Adipose tissue, or fat tissue, can now be classified as an endocrine organ as it responds to stimuli by secreting a range of hormones, termed adipokines, which regulate the functions of various other tissues and organs. Because novel adipokines continue to be discovered and characterized by researchers, there is an enduring need for the development of new analytical assays that target these hormones. Discovered recently, asprosin is an adipokine hormone secreted by white adipose tissue (WAT) during fasting which has been implicated for its important effects on the liver, skeletal muscle, hypothalamus, pancreas, and possibly other tissues. While standard immunoassays have been developed, the continued surge in research on asprosin's function would greatly benefit from an assay with homogeneous, mix-and-read workflow, and the nanomolar clinical range makes this goal more feasible. In this work, we developed such an assay for asprosin using our thermofluorimetric analysis (TFA) methods with antibody–oligonucleotide conjugate probes. The assay, achievable in less than one hour, was successfully validated by quantifying native levels of asprosin in human serum collected from fasting, nonfasting, type II diabetic, and obese donors.

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Introduction

Quantitative sensing is critical to understand a particular protein's functional role in physiological and pathological processes. Current methods can be classified into two categories: homogeneous or heterogeneous. In heterogeneous assays, such as enzyme-linked immunosorbent assays (ELISA), reagents are added in sequence, and several washing steps are required to avoid the false positive results and improve detection sensitivity, often including additional instrumentation such as plate washers. Although high sensitivity can be achieved by the enzyme amplification effect, the expense, complicated workflow, and time-consuming procedures required in these assays place constraints on the efficiency of research. These constraints are particularly detrimental for characterizing the effects of newly discovered proteins or hormones. In contrast, homogeneous assays are defined by simple protocols (*e.g.* mix-and-read), they are typically lower in cost, and most are amenable to miniaturization and automation. With microfluidic systems becoming more powerful as tools for biological enquiry at high temporal and spatial resolution,[1–3] the development of compatible assays is critical.

Several homogeneous assay formats have been developed, such as the AlphaLISA, although specialized equipment is needed.[4] One widely exploited approach is to develop assays based on DNA-driven assembly to measure proteins and small molecules, where the quantities of target molecule are translated to the readout of DNA hybridization.[5,6] Proximity ligation assay (PLA) and proximity extension assay (PEA) are two typical assays using DNA assembly and downstream enzymatic amplification, and these are well developed and highly sensitive.[7,8] However, these assays require ligation and polymerase chain reactions, tight temperature control, expensive reagents, and a relatively tedious protocol. Another way to quantify the proximity-induced hybridized DNA[9–11] is *via* fluorescence quenching or fluorescence excitation by Förster resonance energy transfer (FRET), such as in the molecular pincer assay developed by the Heyduk group.[12–15] However, background fluorescence or FRET signals in these assays cannot be easily distinguished from the analyte-dependent signals.

In our previous work, we have leveraged thermofluorimetric analysis (TFA) to analytically separate such signal and background,[5] and this approach was shown useful for aptamer-based assays,[9,16] antibody–oligonucleotide-based assays,[6,9,17,18] and even small molecule assays based on DNA-binding proteins.[16] The TFA assay methodology is based on the different melting temperatures of double-stranded DNA reporters which are attached to probes such as aptamers or antibodies, where analyte-stabilized complexes (signal) exhibit a higher melting temperature than analyte independent

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complexes (background). This approach is advantageous due to its simple procedure, *i.e.* a mix-and-read workflow, showing rapid readout of about 0.5–1.0 hour with limits of detection (LODs) in the sub-nanomolar range.[5] Using thermal scanning with a standard qPCR instrument and differential data analysis, TFA can function well in complicated sample matrices such as culture media, serum, or plasma, and the same standard curve can be shown to perform consistently over several months.[16] Herein, we have developed a TFA based sensor for a newly discovered protein, asprosin.

Asprosin is a recently discovered protein hormone, identified in by Chopra and coworkers.[19,20] It is a fasting-induced hormone, mainly secreted by white adipose tissue (WAT), and it was shown to strongly regulate hepatic glucose production. Subsequently, asprosin was proven to be an orexigenic hormone that stimulates appetite through the activation of Agouti-related protein (AgRP).[21] Similar to ghrelin, asprosin was suggested to be a potential therapeutic target in post-burn treatments,[22] and circulating levels of the hormone—present in nanomolar concentration in blood—have been linked to carbohydrate disturbances,[23] triglyceride levels,[24] and browning of WAT.[25] The hormone has also been linked to estradiol and progesterone secretion from granulosa cells[26] and to obesity in children.[27] While its importance as a potential therapeutic target for obesity, diabetes, or metabolic syndrome is becoming clear,[19–40] typical laboratory measurements of asprosin are done by the laborious and expensive ELISA method using kits available from a few commercial sources, and highly variable results have been shown with such commercial assays.[36] Others have pointed to the need for better assays or to better understanding of the distribution of asprosin in various bodily fluids,[30] and recent efforts to address these concerns have introduced improved sandwich ELISA assays, affinity chromatography sample preparation, and even correlation with surface plasmon resonance (SPR).[36]

In this project, we designed customized antibody–oligonucleotide conjugates for human asprosin binding and investigated their use in a TFA based assay for quantifying asprosin *via* a simpler, mix-and-read workflow compared to other available assays. This novel TFA was applied to sensing native levels of the hormone in human serum collected from donors with different fasting statuses. This approach provided the first demonstration of homogeneous, mix-and-read quantification of asprosin in less than 1 hour in human serum samples, and the method demonstrated high selectivity for the human version of the hormone. This new assay format should be useful for other groups striving to delineate the endocrine role and the distribution of asprosin in the human body.

Materials and methods

Reagents and materials

The asprosin antibody pair was chosen from fibrillin 1 commercially available antibodies to recognized different amino acid sequences of asprosin. Anti-fibrillin 1 (FBN1) monoclonal antibody (aa2772-2872) was obtained from antibodies-online (product#: ABIN396145), and FBN1 polyclonal antibody (catalog#: PAB17824) was purchased from Abnova. These two antibodies were conjugated to single-stranded DNA oligonucleotides and purified by Mediomics (St. Louis, MO). "Oligo MANT" was linked to the monoclonal antibody, with the DNA sequence obtained from IDT as follows: 5′-/5AmMC6/TAGGTGCTCGACGCTGAC. "Oligo MAGS" was covalently bound to the polyclonal antibody, and this sequence is as follows: 5′-/5AmMC6/TAGGAGAGAGAGAGAGGA. The labelled DNA oligo complementary to oligo MANT is "rev-MANT-TAMRA" with a sequence as follows: 5′-GCTCATXGT-CAGCGTCGAGCACCTA (X = dT-TAMRA). The oligo complementary to oligo MAGS is termed "rev-MAGS-FAM" with this sequence: 5′-/6-FAM/ATGAGCTTCCTCTCTC TCTCTCCTA. The conjugated DNA strands and labeled DNA oligonucleotides were obtained from Integrated DNA Technologies (IDT) and were purified by HPLC. 200 μg mL^{-1} recombinant human asprosin (carrier-free) and recombinant mouse asprosin (carrier-free) were purchased from Biolegend (San Diego, CA). Recombinant mouse fibrillin-1/asprosin (N-8His) was obtained from Novoprotein (Summit, NJ). Bovine serum albumin (BSA), HEPES (4-2-hydroxyethyl-1-piperazineethane sulfonic acid), sodium chloride, calcium chloride dihydrate, potassium chloride, and magnesium chloride hexahydrate were purchased from Sigma-Aldrich (St. Louis, MI). DMEM (no glucose, no glutamine, no phenol red, cat#: A1443001) was obtained from ThermoFisher. Human serum samples were collected and sold by BioIVT, where they were pre-screened for viral and bacterial pathogens.

Probe preparation

After purified oligo-conjugated antibodies were obtained from Mediomics, the probe "Asprosin-MANT-TAMRA" (Probe A) was prepared by mixing the 2.90 μM oligo MANT-conjugated polyclonal antibody and 9.00 μM rev-MANT-TAMRA oligo at equivalent volumes, then incubating for 30 min at room temperature. Similarly, 4.70 μM oligo MAGS-conjugated monoclonal antibody and 4.81 μM rev-MAGS-FAM oligo were mixed at equivalent volumes and incubated for 30 min at room temperature to generate the probe "Asprosin-MAGS-FAM" (Probe B).

Thermofluorimetric analysis (TFA)

Probe A and B were diluted from each stock to 60 or 80 nM for probe premix, four-fold higher than the probe concentration in the final assay solution. Dilutions were accomplished with BMHH buffer, consisting of 0.1% BSA, 10 mM HEPES, 125 mM NaCl, 5.7 mM KCl, 2.5 mM $CaCl_2$, and 1.2 mM $MgCl_2$. Based on the probe concentrations, eight or nine different concentrations of recombinant human asprosin were prepared for the standard curves from the stock asprosin solution, which was diluted in BMHH buffer with 0.1% BSA. Probes A and B were mixed to make the probe premix. Then, 10 μL of assay probe solution and 10 μL of asprosin standard were mixed and incubated at room temperature for 30 min. The final assay solution was loaded into a 96-well qPCR plate for measurements. A real-time quantitative PCR (qPCR) instrument, the Bio-Rad CFX96, was used for all experiments. The protocol was set to incubate assay mixtures at

4 °C for 10 min, then the tubes were scanned thermally from 4 °C to 90 °C with a 0.5 °C increment and a 2 second delay before fluorescence emission was measured. During thermal scanning, the FAM channel ($\lambda_{ex} = 470 \pm 20$ nm, $\lambda_{em} = 522 \pm 8$ nm) was chosen for fluorescence quantification for each solution. The solution with maximum fluorescence was prepared by mixing the 4× probe B and 15 μL BMHH buffer with 0.1% BSA, which was used for the normalization in data analysis. The solution of minimum fluorescence was 20 μL BMHH buffer with 0.1% BSA used to exclude the background optical signals from the assay buffer. Triplicate experiments were done for the standard curves and quantifications of asprosin.

Asprosin measurement in human serum

Different dilutions of serum samples were applied to give a total of 10 μL of each sample for analysis. 5 μL serum and 5 μL BMHH buffer with 0.1% BSA were mixed to prepare the sample solutions from non-fasted and type II diabetic, while 6 μL serum and 4 μL BMHH buffer with 0.1% BSA were mixed for obese donors. For the human serum from fasted donors, 3 μL human serum and 7 μL BMHH buffer with 0.1% BSA were mixed to prepare the samples solution. 10 μL assay probe solution and 10 μL of these diluted samples were then mixed and incubated on ice for 30 min, after which thermal scanning was done using the qPCR instrument as described above. A modified solution with maximum fluorescence (for normalization) was prepared by mixing 5 μL of 4× probe B, the appropriate volume of human serum (5, 5, 6, or 3 μL for non-fasted, diabetic, obese, or fasted, respectively), and enough BMHH buffer with 0.1% BSA to reach a total of 20 μL per sample. Solutions with minimal fluorescence were made without probe using serum (5, 5, 6, or 3 μL) and buffer volumes (15, 15, 14, or 17 μL) required for the sample type (non-fasted, diabetic, or fasted).

Statistical analysis

For statistical analysis, data was processed using the unpaired Student's *t*-test with heterogeneous variance in asprosin measurements of human serum.

Results and discussion

TFA data analysis and asprosin standard curve

The overall probe design for this novel asprosin assay (Fig. S1†) was inspired by the molecular pincer assay,[12] which is a homogeneous immunoassay. The tails of probe A and B were designed to hybridize with each other through a small (weaker) segment, such that background is less likely to form compared to analyte-driven signal. In the presence of asprosin, the fluorescence of FAM in probe B will be quenched by TAMRA in probe A based on FRET quenching. During scanning from low to high temperature, the assembled DNA will denature, and the fluorescence will increase as the separation of fluorophore and quencher occurs. The raw fluorescence data was measured by the qPCR instrument during thermal scanning, and the data analysis for the asprosin TFA assay is shown in Fig. 1.

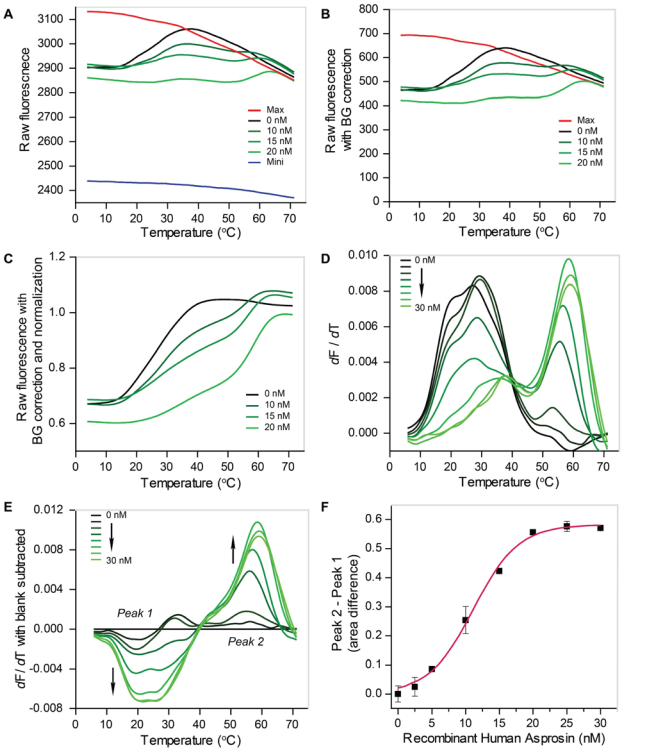

Fig. 1 Data analysis and calibration for the asprosin TFA assay. (A) Raw fluorescence data from qPCR instrument with different recombinant human asprosin concentrations is shown along with the (B) background-corrected data, the (C) normalized data, (D) the derivative data, (E) the blank-subtracted derivative data, and (F) the asprosin calibration curve based on peak area differences, with a 3-parameter sigmoidal fit. Error bars represent standard deviations on triplicate measurements.

In these experiments, 20 nM of probe A and B were used to bind asprosin. The raw fluorescence data were plotted in Fig. 1A against the concentration of recombinant human asprosin. Due to the background fluorescence from the detection system and buffers used in the assay, the minimum fluorescence from the buffer (Mini, lower blue curve) was used to exclude the background fluorescence by subtracting it from the raw data at each temperature (Fig. 1B). Because the fluorescence emission of the fluorophore decreases as the temperature is increased, the maximum fluorescence curve (red) from 20 nM of probe B was used to correct the data further by normalizing the resultant data as shown in Fig. 1C. After background correction and normalization, it was clearly demonstrated that the fluorescence increases during melting, and that the pattern was dependent on asprosin concentration. Two different melting transitions were observed, similar to the thrombin, insulin, and cyclic AMP assays described in our previous TFA work.[5,6,9,16] This result is attributed to the proximity effect, in that the target binding will bring the DNA tails close to each other for hybridization and help stabilize the assembled DNA helix at the probe tails, thus the melting of the DNA helix will shift to higher temperatures in the presence of asprosin to give a new, higher-temperature melting transition.

When the first derivatives of the data from Fig. 1C were taken (dF/dT), the melting temperatures could be more easily obtained for the two different complexes, with or without target binding, as exhibited in Fig. 1D. In this figure, two different melting peaks are shown, the lower-temperature peaks (20–30 °C) representing dissociation of the background complex (without asprosin) and the higher-temperature peaks (53–59 °C) representing the asprosin-stabilized signal complex. It is noteworthy that the peak maxima shifted slightly shifted as the asprosin concentration was changed, signifying a complex temperature-dependent equilibria.

To achieve enhanced visualization of the two melting peaks, the blank was then subtracted from these data, with the result shown in Fig. 1E. Peak 1 in this plot represents the asprosin-dependent disappearance of unbound background complexes, while Peak 2 represents the growing of signal complexes as a function of asprosin concentration. The differences in peak areas—which are proportional to the amounts of each complex—were then used as the assay response for asprosin quantification, and the standard curve is plotted in Fig. 1E. Using a 3-parameter sigmoidal equation as shown below

$$y = a/(1 + e^{(x_0 - x)/b})$$

the standard curve was fit to the peak area difference data using nonlinear regression (red line in Fig. 1F), giving an $R^2 = 0.9967$. In this fit, the values were as follows: $a = 0.5805$, $b = 3.123$ and $x_0 = 11.1641$. The 3σ limit of detection (LOD) of this asprosin TFA assay was 4 nM, and the dynamic range of detection was from 4 to 20 nM. It should be noted that the dynamic range can typically be tuned somewhat by manipulating the probe concentrations.

Dependence on probe concentration

To lower probe concentration and reduce the assay cost as much as possible, different concentration of probes were tested in preliminary experiments (not shown), with the optimal results using 15 nM shown in Fig. 2 alongside the data with 20 nM probes. The data with 15 nM probes was also regressed by applying 3-parameter sigmoidal equation, where $R^2 = 0.9956$, $a = 0.5693$, $b = 2.5898$, and $x_0 = 9.9267$. For 15 nM probes, the detection range also extended up to 20 nM. While slightly better assay performance was given using 20 nM of probes, when the errors at each concentration were taken into account, the LOD and the detection range were similar using both probe concentrations. Thus, the 15 nM probe concentration was chosen for later experiments. It is well-known that the standard 3σ method for LOD calculation is dependent on the standard deviation in the blank without asprosin in the assay solution, and later data showed that the LOD of our asprosin TFA assay can be reduced down to 0.6 nM using 15 nM of probes, after the assay condition was later modified by changing the BMHH buffer to DMEM media to test cell secretion samples (Fig. S2†).

Specificity test of asprosin TFA

To test the specificity of this assay, recombinant mouse asprosin and recombinant mouse asprosin with 8-His tag were utilized to perform the same type of experiments, and these results are depicted in Fig. 3. The accurate 3D structures of human and mouse asprosin are unknown at this point, but the mouse asprosin has 91% amino acid sequence identity compared to human asprosin, according the Protein BLAST online software. Since the assay responses to either mouse asprosin were negligible over the 0 to 30 nM range, these data proved that our asprosin TFA assay was characterized by high specificity toward human asprosin. Such high specificity is likely a result of using a combination of two antibody–oligo conjugated probes. Even though one was a polyclonal antibody and the other was monoclonal, the dual sire binding requirement introduces a high level of stringency for target identity.

Quantifying asprosin in human serum

The newly developed asprosin TFA assay was next validated by measuring asprosin in human serum. Four different groups of

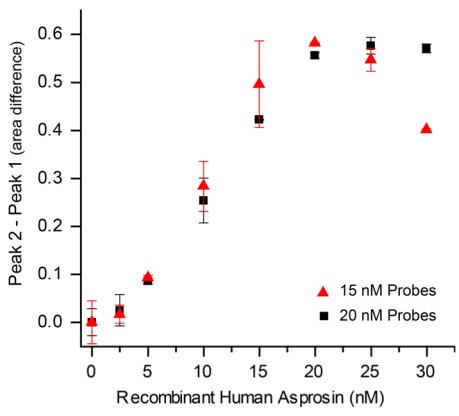

Fig. 2 Standard curves for asprosin TFA assay with two different concentrations of probes, 15 nM and 20 nM. Triplicate experiments were run for each data point, with error bars representing standard deviation.

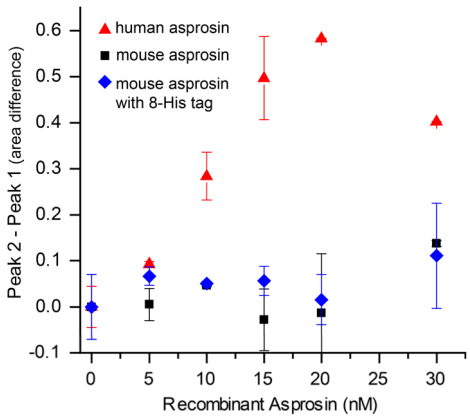

Fig. 3 Specificity test for the asprosin TFA assay. The assay response to two similar proteins, recombinant mouse asprosin and recombinant mouse asprosin with 8-His at the N-terminus, were compared to that with human asprosin. Triplicate experiments of human asprosin were compared to duplicate experiments with the other two proteins, and error bards report standard deviations.

patients were sampled (by the commercial provider, BioIVT), including fasting, non-fasting, obese (Body Mass Index (BMI) >30), and type II diabetic donors. Between 4 and 5 different patients' serum samples were collected for each group. In our previous publications,[6,9,16] TFA was proven to perform well in human serum and plasma, since the differential analysis could largely eliminate autofluorescence influences from the complex matrix. Similarly, as shown by the results in Fig. 4, the thermofluorimetric assay was successfully applied to detect asprosin directly in human serum. The asprosin concentration in the serum from fasted donors were significantly higher than those in the samples from non-fasted donors, as expected. According to the seminal publication,[19] asprosin was characterized as a fasting-induced hormone, so fasting conditions should boost the asprosin concentration in human serum, as observed here.

The data from obese and T2D group were less obvious to interpret, due to the unknown information about the fasting status of the donors. In our experiments, the obese and diabetic donors showed significantly lower asprosin levels than healthy donors. Further studies are required in the future, particularly since others have pointed to difficulties in correlating asprosin measurements under varying diseased states.[30,36] In compiled and tabulated data, Morcos and coworkers[36] showed that asprosin concentrations measured in blood samples from children showed large variations, up to four orders of magnitude (<1 ng mL^{-1} up to >100 ng mL^{-1}). They also showed that the assay responses of standard ELISA kits vary more than an order of magnitude, giving standard detection ranges from 5 pM to 330 pM in one instance and from 0.3 nM to 17 nM in another. Detection limits varied over an order of magnitude as well.

While more work is required, on the other hand, one aspect of our results is encouraging, namely the fact that the variance of measurements in each group of donors was relatively low, suggesting some convergence of asprosin levels under each physiological state. Overall, these results suggest that our novel, easy-to-use asprosin TFA methodology could be a valuable addition to the toolbox of physicians seeking to evaluate the health of their patients, particularly those showing compromised glucose homeostasis. The TFA method should also be useful to scientists looking to further study the biological effects of asprosin. Although the overall asprosin concentrations measured in human serum were higher in our study compared to the original paper,[19] other publications have shown circulating asprosin comparable to our measurements.[41]

To further validate our TFA method's performance, human preadipocytes were cultured and differentiated according to the manufacturer protocol (Fig. S3†) and used for asprosin secretion tests. The results showed that asprosin secretion increased by 2–3 fold in DMEM media without glucose compared to in the media with 4.5 g L^{-1} glucose (Fig. S4†). Therefore, our TFA method was successfully proven with recombinant asprosin standards, human serum samples, and with human cell secretions.

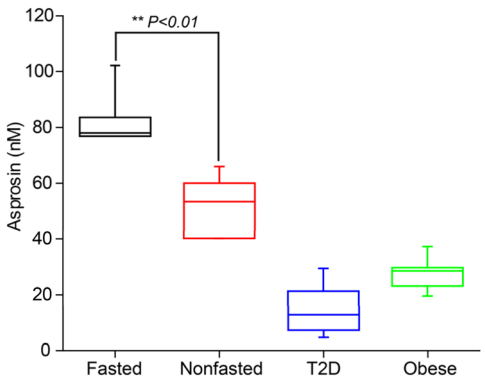

Fig. 4 Clinically-relevant application of our asprosin TFA assay. Serum samples from four different groups of patients were measured: fasted, nonfasted, type II diabetic, and obese (with Body Mass Index >30). Each group was represented by 4–5 different patient serum samples. Duplicate experiments were run for each sample. Expected differences were observed in fasting and nonfasting states, while the T2D and obese samples were novel under these conditions.

Conclusions

A direct, fluorescence based, mix-and-read proximity assay format was chosen to design antibody–oligo probes for human asprosin. With these probes, our thermofluorimetric analysis (TFA) approach was successfully applied as an asprosin assay with sub-nanomolar LOD, and the assay was validated in buffer, in human serum samples, and in human cell secretion samples. Considering that asprosin is a newly discovered adipokine with nanomolar concentration in human circulation system, and that the hormone was shown by other research groups to be both a promising therapeutic target and possibly a therapeutic molecule, this homogeneous assay should be particularly useful in the characterization of asprosin in regulating glucose homeostasis.

With these results, we also showed once again that our TFA assay format can be easily translated to quantify other proteins with the benefits of rapidity, low cost, and simple manipulation.[6,9,16] It is also noteworthy that the derivative data in Fig. 1D shows that background and signal can be clearly separated around 37 °C, suggesting that the assay should function for direct readout at biological temperature without thermal scanning—albeit without the ability for differential background correction. In the way, the asprosin assay may be useful for dynamic research of asprosin secretion from WAT by interfacing to microfluidic droplet-based systems developed in our laboratory[3,17,18,42] or in future applications in cell secretion monitoring or studies in living animals or humans.

Data availability

Data for this article, including raw data for Fig. 1–4 and supplementary figures, have been made freely available[43] at the Harvard Dataverse and can be accessed using the following link: **https://doi.org/10.7910/DVN/FYGO0U**.

Author contributions

J. H. participated in conceptualization of assay design and applications, formal analysis of data, investigation, analytical

methodology development, acquisition of reagents and resources, validation of methods and results, visualization of the results, writing of the original draft, and writing review and editing. C. J. E. participated in overall project conceptualization, formal analysis, funding acquisition, investigation, methodology, project administration, acquisition of resources, supervision of experiments and analysis, visualization of the results, and writing review and editing.

Conflicts of interest

There are no conflicts to declare.

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