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Thermal Measurement Techniques in Analytical Microfluidic Devices

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Abstract:	Thermal measurement techniques have been used for many applications such as thermal characterization of materials and chemical reaction detection. Micromachining techniques allow reduction of the thermal mass of fabricated structures and introduce the possibility to perform high sensitivity thermal measurements in the micro-scale and nano-scale devices. Combining thermal measurement techniques with microfluidic devices allows performing different analytical measurements with low sample consumption and reduced measurement time by integrating the miniaturized system on a single chip. The procedures of thermal measurement techniques for particle detection, material characterization, and chemical detection are introduced in this paper.
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Title:
Thermal Measurement Techniques in Analytical Microfluidic Devices

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Short Abstract:
Here, we present three protocols for thermal measurements in microfluidic devices.

Long Abstract:
Thermal measurement techniques have been used for many applications such as thermal characterization of materials and chemical reaction detection. Micromachining techniques allow reduction of the thermal mass of fabricated structures and introduce the possibility to perform high sensitivity thermal measurements in the micro-scale and nano-scale devices. Combining thermal measurement techniques with microfluidic devices allows performing different analytical measurements with low sample consumption and reduced measurement time by integrating the miniaturized system on a single chip. The procedures of thermal measurement techniques for particle detection, material characterization, and chemical detection are introduced in this paper.

Introduction:
Three different micro-scale thermal measurement techniques are presented in this article. The three different configurations of microfluidic devices are used for thermal particle detection (TPD), thermal characterization (thermal conductivity and specific heat), and calorimetric detection of chemical reactions and interactions.

Thermal Particle Detection.
Detecting and counting particles in microfluidic devices is widely used for environmental, industrial, and biological applications[1]. TPD is one of the novel applications of thermal measurements in microfluidic devices[2]. Using heat transfer for detecting and counting particles based on the particle size reduces the complexity, cost, and size of the system. In other methods, complex optics or complex electrical measurements and advanced signal processing software are used for detecting particles.

Thermal Characterization of Liquid Substances Using Micro-Calorimeter.
Liquid sample thermal characterization is the second application of thermal measurement in microfluidic devices. Performing micro-scale calorimetry will reduce the sample consumption and increase the precision by offering higher repeatability compared to conventional, bulk calorimetry methods. The procedures for thermal conductivity and specific heat measurement using the on-chip micro-calorimeter device are presented elsewhere[3]. The details of the heat penetration time technique for thermal conductivity measurement and the thermal wave analysis (TWA) for specific heat measurements in microfluidic devices are described in the protocol section.

Calorimetric Bio-Chemical Detection in Paper-Based Microfluidic Device.
Another application of thermal measurement is biochemical detection in paper-based microfluidics. The capillary action in the porous structure of paper carries the liquid and avoids bubble initiation problems in micro-channels. The most common detection mechanisms in paper-based microfluidic devices are optical or electrochemical techniques. Optical detection suffers from high complexity and the necessity of advanced image processing software to quantize the detected signal. Electrochemical detections are also limited because they can only be applied to reactions that produce active byproducts. The recently introduced calorimetric paper-based biochemical sensor platform[4] takes advantage of the paper-based microfluidic system and the label-free thermal detection mechanism. The procedures of calorimetric detection of glucose using glucose oxidase (GOD) enzyme in a paper-based microfluidic platform are presented in the protocol section.

The goal of this paper is to demonstrate the capabilities of thermal measurement techniques in microfluidic devices. The device preparation, liquid sample handling and resistance temperature detector (RTD) sensor excitation and measurement are presented in the next sections.

Protocol:

1. Thermal Particle Detection (TPD)

1.1. Prepare the micro-fabricated silicon device with a thin-film silicon nitride membrane and integrated temperature sensor by micromachining, using standard semiconductor processing technology[2]. Rinse the fabricated device with deionized (DI) water.

Note: The fabrication method for thermal particle detector microfluidic device is explained in prior publication[2].

1.2. To produce polydimethylsiloxane (PDMS) substrates with micro-channels, create an SU8 mold using standard lithography processes[5].

Note: The channel size is designed for each specific particle's dimension.

1.2.1. Make PDMS by mixing a 10:1 ratio of base (30 mL) and curing agent (3 mL). Pour the PDMS on to the mold and remove the bubbles by briefly exposing it to a vacuum (5-10 min).

Note: The vacuum level is not a critical value to the degasification and it should continue until gas bubbles are totally removed from mixed PDMS.

1.2.2. Place the mold on a hotplate (≈70 °C) for 2 hours to cure the PDMS. Then peel off the PDMS very carefully so as not to damage the mold.

Note: The Vacuum level is not a critical value.

1.3. Using a manual punch, punch a tight hole (1 mm) for the PTFE tube at one end. Use a large punch (2 mm) at the other end to make the PDMS a reservoir. Place the punched micro-channel on top of the device under the microscope and align the RTD at the center of the micro-channel (Figure 1A).

1.4. In the electrical interface, connect the electrical pins at the contact pad positions and tighten up the locking screws. Make sure the height-adjustable pins (Pogo pins) sit at the correct electrode pads on the device.

1.5. Dilute 10 µL of the concentrated PS beads in 100 µL of DI water in a 1.5 mL tube.

1.6. To ensure the PS beads remain neutrally buoyant, add 2.7 µL of glycerol (1.26 g/cm^3) to DI water to match the fluid density to the polystyrene (PS) bead density (1.05 g/cm^3).

1.7. Connect the PTFE tube to the channel at one end and the other end to a 1 mL glass syringe. Fill the glass syringe with 0.5 mL of DI water.

Note: Tight fitting made by selecting the right punch size will avoid leakage in tubes.

1.8. Place the DI water filled syringe on the computer-controlled syringe pump. Push the water (5-20 µL/min) into the channel to fill the whole channel with fluid all the way to the reservoir.

1.9. Load 10 µL of balanced bead solution to the reservoir and introduce the bead solution to the micro-channel by changing the flow direction on syringe pump.

1.10. Turn on the RTD by biasing 1 mA of DC current through the computer controlled source/meter while measuring the resistance by source/meter and sorting the measured data (Figure 2).

Note: During the experiment, the sensor is biased; therefore, the temperature is continuously measured until the end of the counting experiment. The RTD sensor is electrically biased by applying a DC current in the range from 100 µA to 1 mA to continuously measure the temperature until the end of the counting experiment. It is critical to select the correct current level since there is a trade-off between noise level and the detected signal amplitude. The syringe pump is used to generate the flow in micro-channel. Selecting an appropriate flow rate to perform the TPD experiment is limited to the speed of the measurement. This speed is a function of the thermal time constant of the device and electrical measurement speed. The results of thermal particle detection experiment are shown in Figure 3.

1.11. Use the developed data processing software (LabVIEW) to convert the measured resistance data to temperature using the Callendar–Van Dusen equation[6].

2. Thermal Characterization of Liquid Substances Using a Micro-Calorimeter

2.1. In this process, use the on-chip calorimeter device (Figure 4A) [3] to measure the thermal diffusivity and the specific heat of the samples.

Note: On each die, there are 2 micro-calorimeter chambers (Figure 4B). Each chamber has 2 inlets and one outlet. And each chamber has a heater and a RTD sensor integrated.

2.2. Place the micro-calorimeter device on the device holder (Figure 4C). Align the device to the microfluidic inlets and outlets with the holder fittings. Place the PDMS seal layer on top of the device.

2.3. Install electrical connection pins on the device holder and lock the holder screws.

Note: Make sure the height-adjustable Pogo pins are aligned with the electrical contact pads.

2.4. Install the microfluidic interface layer with magnetic latches to the device holder (Figure 4D). Connect the PTFE tubes to both inlets and the outlet. Connect one inlet to the sample-loaded syringe pump and close the other one, as the enthalpy is not measured in this case.

2.5. Use a developed computer-controlled program to load the sample into the micro-channel and chambers.

Note: The program will use discontinued flow to release excessive pressure on the thin-film suspended chamber.

2.5.1. Load the 300 µL sample into the glass syringe and place it on the syringe pump. Use very slow (0.25 µL/min) constant flow rates for high viscosity samples (*e.g.*, glycerol and ionic liquids). Use a glycerol sample for thermal diffusivity measurements and ionic liquids for specific heat measurements.

2.6. For thermal diffusivity measurements, connect the measurements setup as shown in Figure 5A. Load the glycerol sample to the micro-calorimeter chamber. Run the modified computer controlled program for heat penetration time measurement.

2.6.1. Use the calibrated heat penetration equation to calculate thermal diffusivity from the measured heat penetration time[7]:

$$\alpha = \left[\frac{(L \times p)^2}{\left(\frac{16}{\pi}\right) t_0} \right]$$

where α is thermal diffusivity, L is thickness of the chamber, p is the thickness calibration factor due to fabrication process variation, and t_0 is heat penetration time.

2.7. For specific heat measurements, use the TWA measurement setup as shown in Figure 5B. Use the same sample loading program and load the ionic liquid in the chamber. Run the TWA program to get the amplitude of the AC temperature fluctuations (∂T_{AC}) and use the specific heat equation to calculate the specific , c_p , heat for each ionic liquid sample[8]:

$$c_p = \frac{C_0 P_{in}}{2\omega m (\partial T_{AC})}$$

where C_0 is input power calibration factor, P_{in} is input power, ω is frequency of the actuation signal, and m is the mass of liquid sample.

3. Calorimetric Bio-Chemical Detection in Paper-Based Microfluidic Device

3.1. Use microfabricated thin film (40-50 nm nickel) RTD sensor. Fabrication steps for the RTD sensor are explained in previous works[4].

3.2. For paper-based channel fabrication[4], use a knife plotter to cut the paper microfluidic channels with a designed pattern (L-shape). Place the paper on top of the cutting mat, load the paper and the cutting mat to the knife plotter, and use the appropriate recipe to cut the microfluidic paper channels[4].

3.3. For device and channel integration, use an acrylic adhesive layer (5 µm) to integrate the paper on the RTD sensor. Use a clean blade to push the paper to the device and remove air bubbles (Figure 6A). The acrylic film is an adhesive layer to hold the paper over RTD sensor.

3.4. For enzyme activation, use 50 mM sodium acetate buffer to activate the GOD enzyme. Add 1 mg of the GOD enzyme to 1 mL of sodium acetate buffer to make the 1 mg/mL solution. Adjust the pH of the solution to 5.1.

Note: Adjust the amount of acetic acid in the sodium acetate buffer to maintain the PH of solution 5.1.

3.5. Bias the RTD with 1 mA of DC current to activate the RTD and start measuring the resistance source/meter continuously while the resistance settles down after the experiment (≈4 min).

Note: Figure 6B shows the measurement setup for the paper-based calorimetric test.

3.6. Introduce the 2 µL of the prepared GOD solution to the center of the paper micro-channel (immobilization site) via pipette. The detected temperature (Figure 7A) must start to decrease.

Note: This cooling effect is due to the higher operation temperature of the RTD and evaporation of the sample together.

3.7. To measure the glucose concentration, introduce standard glucose control solution[9] to the channel inlet and measure the resistance change caused by the reaction. Repeat this experiment with all different glucose control solutions (high, normal and low concentrations) and save the resistance data.

3.8. Using the temperature coefficient of resistance (TCR) for nickel RTD and Callendar–Van Dusen equation, convert the resistance change to the temperature. Calculate the concentration of the glucose in each sample by considering the reaction enthalpy of glucose and the GOD enzyme ($\Delta H = -80$ kJ/mole) and using the concentration equation[10]:

$$n_p = C_p \frac{\Delta T}{\Delta H}$$

where n_p is detected molar concentration, C_p is heat capacity of the system and ΔT is calculated temperature.

Results:

Figure 3 shows the plot of the measured thermal signal. The generated signals in the presence of the beads with corresponding optical images show the successful detection of the microsphere PS beads in the micro-channel. The thermal conductivity of the liquid passing through the micro-channel is changing due to the presence of PS beads. This change in the thermal conductivity of the channel is affecting the heat transfer in the micro-channel. The change in the heat transfer in the micro-channel is detected by RTD in the form of resistance fluctuation (Figure 3 A and B).

The detected signal can also be affected by the change in the local flow field (Figure 3 C and D), which will affect the heat transfer in the channel. The change in the thermal conductivity will increase the temperature. Furthermore, the local velocity changes in the micro-channel based on the comparable dimensions of the PS bead to the channel size, causing an increase in local heat transfer. In this case, the effect of change in heat transfer is dominant as it appears as a decrease in detected resistance. Therefore, the correspondence of channel size with particle size is essential in TPD experiment. The present results demonstrate the capability of the TPD technique to count and detect the size of particles.

The measured value of thermal diffusivity of glycerol is 9.94×10^{-8} m^2/s, which is within 8% of the theoretical value. Table 1 shows the measured values of different ionic liquid samples by the introduced method. To verify the accuracy of the measurement, the specific heat of water was measured using the same technique with less than 5% error.

The detected temperature signal due to the exothermic reaction of glucose and GOD is shown in Figure 7A. The reaction area on the designed micro-channel is 45% of the total area. To calculate the concentration, only this portion of glucose will be considered. The finite rate of the glucose oxidation reaction is also considered as a reaction kinetics factor. Comparing the detected concentration with available commercial glucose meter results (Figure 7B) shows higher precision (<30%) in the fabricated device**.**

Figure 1. Microfluidic device for thermal particle detection. **A)** Device schematic. **B)** Cross-sectional view of the particle detection using the thermal measurement method.

Figure 2. The experimental setup for the thermal particle detection (TPD). A computer-controlled source/meter is used to bias the RTD and measure the resistance.

Figure 3. Results of thermal particle detection. **A)** The detected resistance change when the 90 um PS bead is passing the RTD sensor with flow rate of 5 µL/min. The explained change in the thermal conductivity will increase the temperature and appear in the form of resistance change in the RTD resistance measurement. **B)** The optical image of the same bead in Figure 3(A) passing the sensor. **C)** The detected resistance change when the 200 µm PS bead is passing the RTD sensor with flow rate of 5 µL/min. **D)** The optical image of the same bead in Figure 3(C) passing the sensor. This Figure has been modified with permission from [2].

Figure 4. The on-chip fabricated micro-calorimeter and the device holder. **A)** A photograph of micromachined 3-dimensional on-chip suspended micro-calorimeter device. The chip has two identical chambers, each of which has two inlets and one outlet. **B)** The schematic of the micromachined micro-calorimeter chamber. The micromachined RTD is shown at the top surface of the fabricated device. **C)** The micro-calorimeter device is placed on the device holder. **D)** The final setup of the micro-calorimeter with electrical and microfluidic connections. The result of TWA is used for the heat capacity calculation. This Figure has been modified with permission from [3].

Figure 5. The electrical connections of the thermal measurement setup with the micro-calorimeter device. **A)** The measurement setup for heat penetration time analysis. The measured heat penetration time is used for thermal conductivity calculation. **B)** The measurement setup for thermal wave analysis. The result of TWA is used for heat capacity calculation. This Figure has been modified with permission from [3].

Figure 6. A) The schematic of the paper-based device. **B)** The measurement setup for paper-based calorimetric detection of glucose. In this setup, a LabVIEW-controlled source/meter (Keithley 2600) is used to bias the RTD and measure the temperature simultaneously. The measured temperature and the time stem will be stored while being measured. In this experiment Keithley 2600 is used for faster measurement.

Figure 7. The glucose detection results with paper-based calorimetric sensor. **A)** Output signal of the glucose and GOD enzyme reaction. **B)** Final detection results of glucose control samples with paper-based device compared with commercial glucose meter results. This Figure has been reused with permission from [4]. "Given Data" is calculated concentration of the glucose in the detection experiments.

Table 1. The measured specific heat of ionic liquids using TWA technique with on-chip micro-calorimeter. This Table has been modified with permission from published data[3].

Discussion:

Different thermal measurement techniques in microfluidic devices and their respective setup procedures are presented in this work. These thermal measurement methods such as thermal conductivity monitoring, thermal penetration time, amplitude of AC thermal fluctuations, and amplitude measurement of the generated heat are used to detect specific substances and investigate different reactions and interactions.

The thermal time constant plays a key role in the aforementioned thermal measurement techniques. In microfluidic device design, the optimization of thermal time constants must be considered. The thermal time constant is a function of the thermal mass and the thermal conductivity of the fabricated device, which are dependent on the material of each component. Using thin-film materials and micro-fabrication techniques allows reduction of the thermal mass of the system. The thermal conductivity is improved by using suspended structures and high thermal conductivity materials to reduce the thermal link to ambient conditions. Also it is important to control the ambient temperature to avoid measurement disturbances by using a thermal isolation.

The thin film RTD offers high sensitivity and linear temperature measurement in the introduced devices over a wide range of temperatures. The thermal and the electronic measurement noises are the constraints for the resolution with the introduced techniques.

Microfluidic devices with thermal measurement methods are capable of performing different physical and chemical measurements within the RTD linear measurement range. These

techniques could also be useful for different chemical and bio-sample reaction and interaction detection for point-of-care applications and sample characterization. The introduced techniques are able to perform measurements from the tissue level to the single cell level.

Disclosure:
No conflicts of interest declared.

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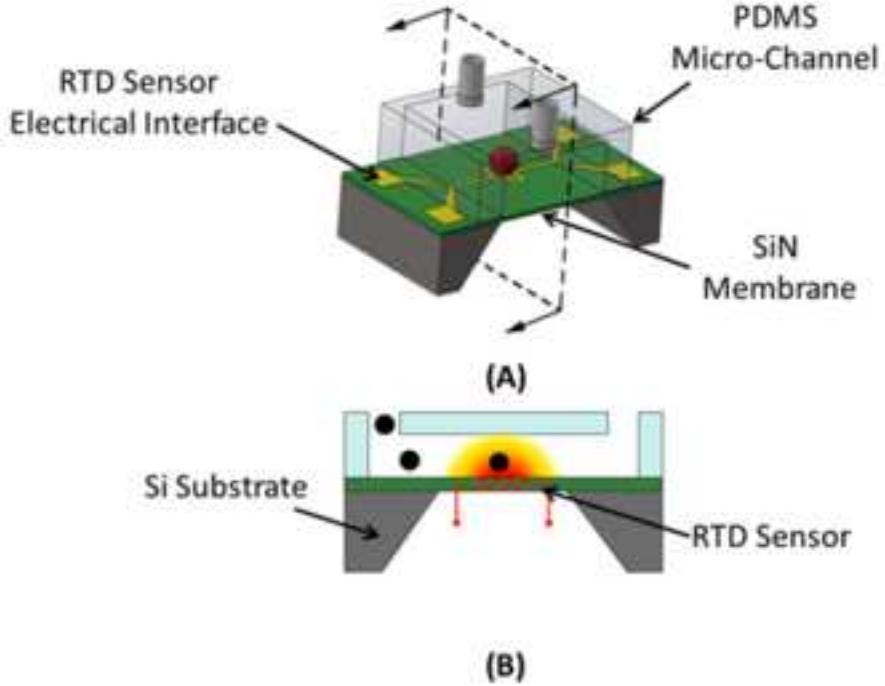

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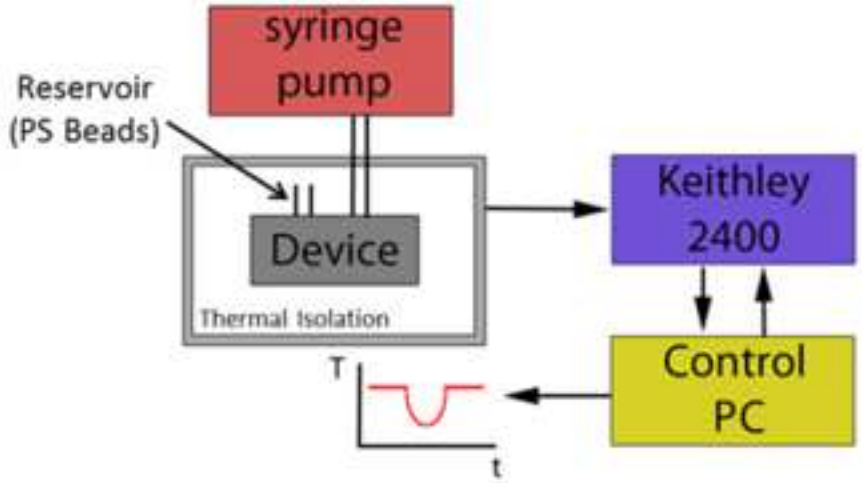

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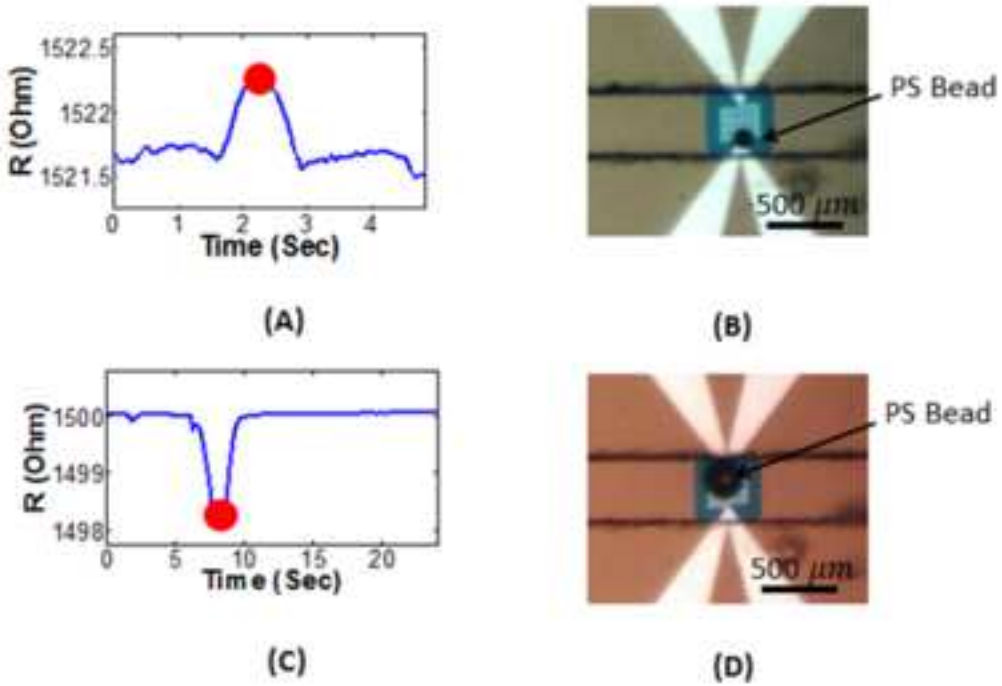

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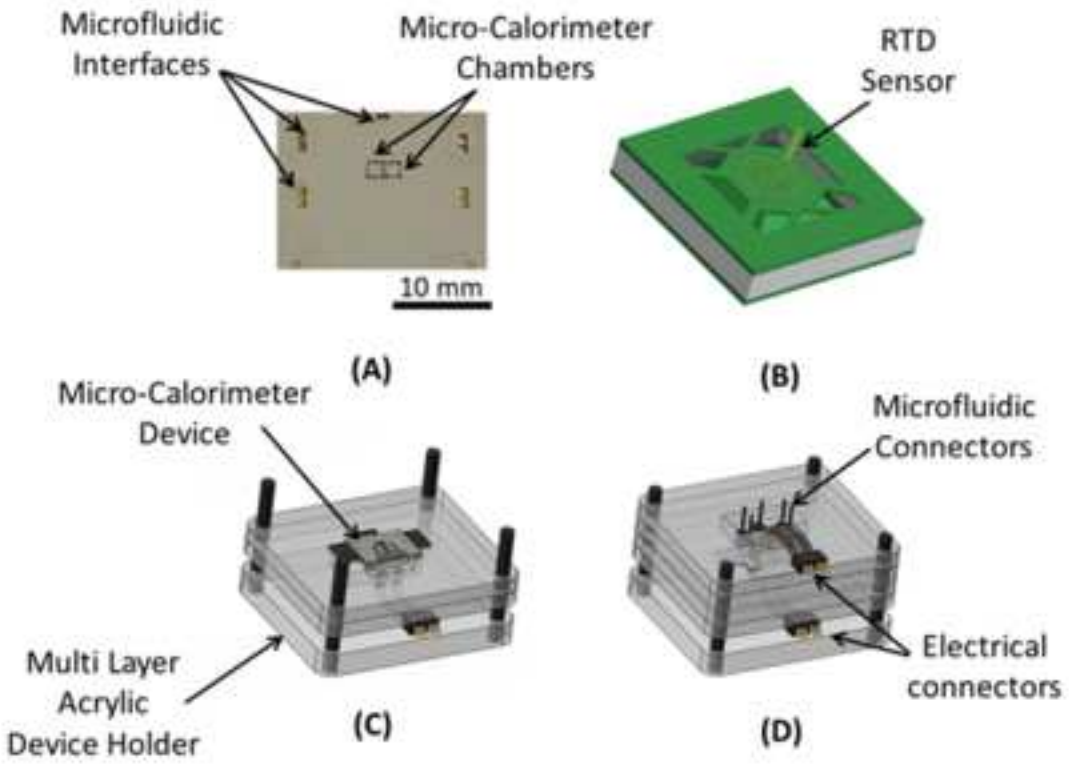

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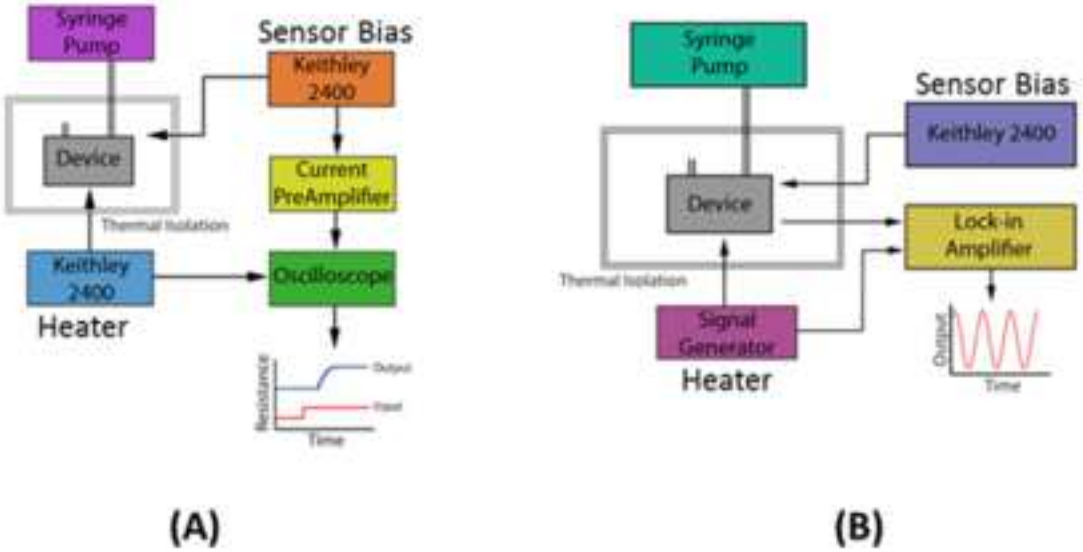

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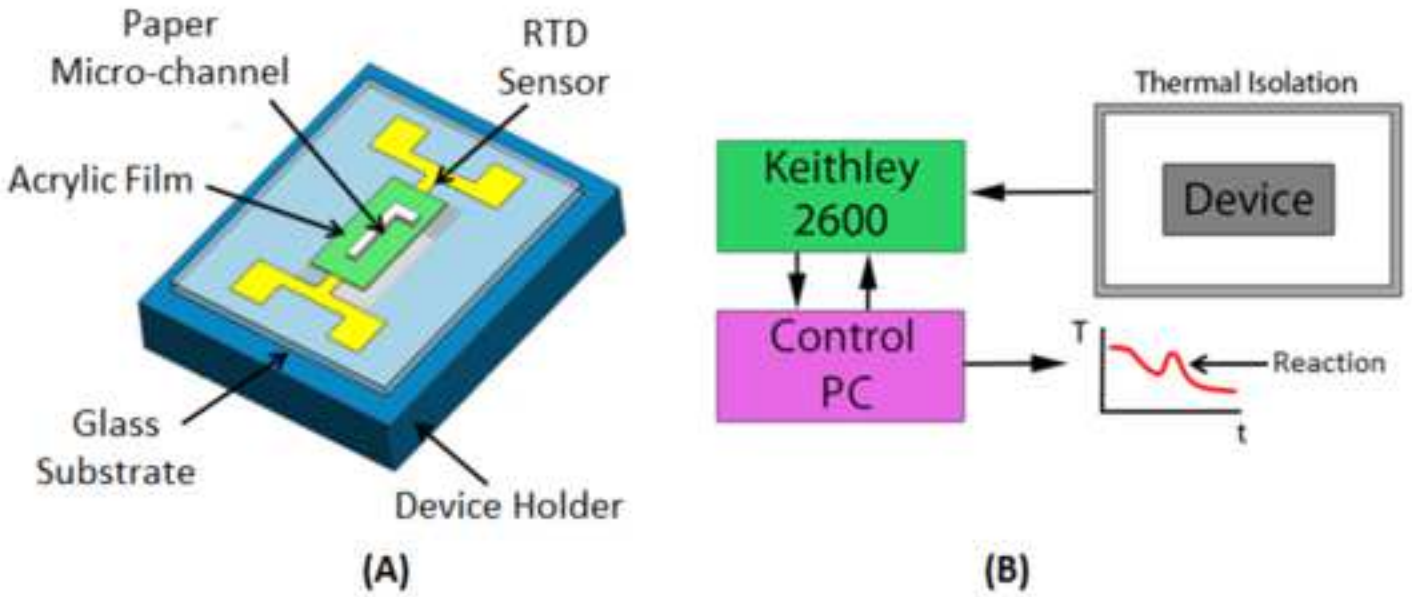

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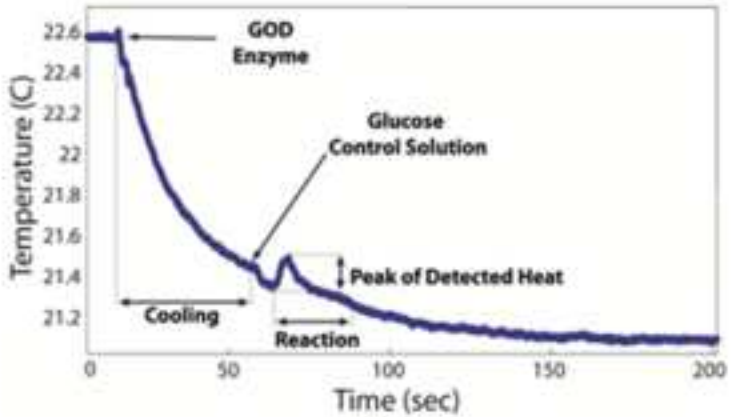

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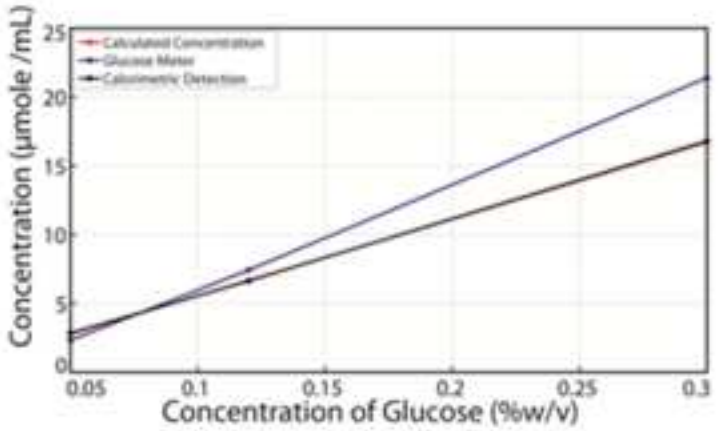

(B)

Excel Spreadsheet- Table of Materials/Equipment

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	Sample	Measured Specific Heat (J/g K)
1	[EMIM][Tf2N]	2.75
2	[BMIM][PF6]	2.83
3	[HMIM][PF6]	0.86
4	[OMIM][PF6]	2.55

Excel Spreadsheet- Table of Materials/Equipment

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Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Polydimethylsiloxane (PDMS)	Dow Corning	Sylgard 184	
PS beads - 90 um	Corpuscular	100265	
PS beads - 200 um	Corpuscular	100271	
Glycerol	SigmaAldrich	G5516	
GOD enzyme	SigmaAldrich	G7141	
Glucose Control Solution-Low	Bayer contour	Low Control	
Glucose Control Solution-Normal	Bayer contour	Normal Control	
Glucose Control Solution-High	Bayer contour	High Control	
Chromatography filter paper	Whatman	3001-845	
Glass	VWR	48393-106	
Acrylic Film	Nitto Denko	5600	
Glass syringe (1 mL)	Hamilton	1001	
Syringe pump	New Era	NE-500	
knife plotter	Silhouette	portrait	
Current Preamplifier	Stanford Research	SR-570	
Ocilloscope	Agilent	DSO 2420A	
Signal Generator	HP	HP3324A	
Lock-in Amplifire	Stanford Research	SRS-830	
Source/meter 2400	Keithley	2400	
Source/meter 2600	Keithley	2436A	

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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
The manuscript is entirely proofread and few corrections are applied and highlighted different color (blue) in the text.

2. In step 1.1 please clarify how to create the device. If it is from the reference, it should state it. Also an image of the device would be helpful.
As suggested, it is clarified that the device fabrication is presented in prior publication [2] in lines 89-90.

3. In step 2.1 - What should the device be used to do?
A brief description for the applications of calorimeter device is added to the lines 154-155.

4. In step 3.4 - How much GOD enzyme is used (concentration)?
GOD enzyme (Sigma Aldrich G7141) is in the form of dry powder. To activate the enzyme, 1 mg of GOD is mixed with 1 mL of sodium acetate buffer (50 mM), as described in the text.

5. Please define RTD with first use.
The definition of the RTD is added to the manuscripts. The mentioned change is in lines 77—78.

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DOIs for all cited journal papers are included in references, as suggested except for reference [7] which was not available.

Reviewers' comments:

Reviewer #1:
Minor Concerns:
Please try to add exact directions to peel the pdms, and the vacuum level needed to pump the bubbles out. Also, simple estimates of the maximum temperature achieved in the microchannel would be useful.
The exact level of vacuum is not essential for degasification; it is added to the text. The operating temperature range of this device is limited by the linear range of Nickel RTD sensor as mentioned in the

text (Line 334).

Reviewer #2:

Major Concerns:
Overall, this manuscript is lacking depth that allows a reader (or viewer) to understand more than the fact that there are microfluidic-based techniques to make these measurements. It seems that one of the main requirements for correctly executing this technique is to have correctly-designed and functioning RTD and microfluidic devices. Because this is a methods publication, with the aim of improving repeatability by visually describing methods, it is not sufficient to simply reference methods described in previous publications without explicitly describing those methods. Therefore, I suggest that the salient device design aspects be discussed, and that methods to ensure proper behavior of the devices be included. In order to dive deeper into the methods, one or more of the particular measurement types may need to be omitted.

Additionally, this manuscript would benefit from added context in terms of other methods or instruments that are available for making similar measurements. What are the benefits to using the microfluidic-based measurements? What are the drawbacks to using microfluidic-based measurements? For what applications are the microfluidics-based measurements best-suited, and what applications or sample types are better characterized using a different method? Also, what does one need to be especially careful of when using the described techniques for thermal measurements? What can lead to incorrect results?

Thank you very much for your deep attention and valuable comments. This manuscript is presenting three different thermal measurement techniques in micro-scale. Due to the nature of this journal, the subject matter primarily focuses on details of experimental method and sample preparation. Benefits of microfluidic device are addressed in various parts of the text body. The key point as it mentioned in discussion section, is considering the thermal time constant of the system and optimizing the design parameters to achieve an acceptable speed and limit of detection.

Minor Concerns:
-There are many typos and mis-spellings in the list of materials and equipment (Sylgard 184, Glucose Control Solution, Pre-amplifier). There are also mis-spellings in the text, so please carefully proofread.
The manuscript is proofread and the mentioned typos as well as other text typos are corrected.

-The list of materials and equipment does not include the RTD devices
The RTD sensors are fabricated on the micromachined devices. We did not use any discrete RTD sensors in the experiments discussed.

-Why are there two different Source/meters used (Keithley 2400 and 2600)?

The Functions of source/meters in the explained thermal measurement techniques are the same. For the paper-based device, we used Keithley 2600 for high speed measurements, as mentioned in the caption of Figure 6.

-Figure 7b has a typo in the legend.
The Figure 7B legend type is corrected.

-Step 2.6.1: how do you determine p (thickness calibration factor)?
The calibration factor is calculated by measuring a sample with known thermal diffusivity. Deionized water is used for calculation of calibration factor for this device. The details are explained elsewhere [3].

-Caption for Figure 4: replace "The real picture" with "A photograph"
The suggested correction is applied to Figure 4 caption.

-More detail in the test setup diagrams would be helpful
The setup diagrams are updated (Figure 2, 5) by adding more information, as suggested by reviewer.

-Please provide example data for Section 2.
The measured data of thermal diffusivity and specific heat are provided in the result section.

Reviewer #3:
Minor Concerns:
It would benefit the reader and flow of the document to have figures appear in order of introduction throughout the paper and to have figure labels. A small introduction of 2-3 sentences to introduce the investigator about the protocol to follow would aid in understanding and cue for expected contents.
The figures with the labels are addressed in order in the text, however. The manuscript format dictates to have figures after the text.

Line 77: please introduce RTD.
The RTD is introduced, as suggested.

1.1 In rinsing the device, is there a specific time/manner the deionized water should introduced to the wafer, i.e. through water bottle in a sheeting action from top to bottom, is there a need for sonication, or post rinse with other substance such as methanol (lower polarity than di-water).
In rinsing, there is no specific time or manner. The only purpose is to remove particles and prepare a clean surface for bonding.

1.2.1 Should the PDMS mold have a specific height or thickness above the sensor or for the reservoir? Is 5-10 minutes sufficient, other groups have suggested up to 1 hour in a desiccator.

The degasification can be visually inspected and be used as soon as the gas bubbles are removed from the PDMS. This time depends on the vacuum level in the desiccator and could vary from a few minutes to several minutes. The procedure for degasification is updated in the text (lines: 100-101).

1.6 It would be beneficial to list a target specific gravity of the final solution that would maintain the beads buoyancy. What about using fluids with higher density? Are there any questions about if a particle can tolerate high density? Is manual mixing of the PS beads acceptable, or use of a magnetic stirring rod?
The target density of the carrier liquid should match the bead density to maintain the buoyancy, as described in this section. It will require further investigation to give precise comment on the buoyancy requirement of this experiment. However, based on the practical data, the matching density with manual mixing will yield in acceptable samples.

1.8 Should the user load the syringe with Di-water?
Yes. This point is added to the protocol in line 127.

1.10 Does the system have any transient effect or hysteresis, does it make a difference if measurements are performed immediately or after some time?
As it is a thermal measurement technique, the speed of measurement is limited by the thermal time constant of the system. This limitation introduces the a measurement lag or in some cases hysteresis and during this lag we will have transient .As explained (lines 329-335), the microfabrication techniques are used to increase the speed of measurement by reducing the time constant of structure, but it will be a finite speed for this technique.

Line 127-133: Suggest moving the notes section to the intro period of this protocol. It has several key pieces of information and a great lead in.
Yes, the mentioned paragraph has key information and the reason we decided to present it after the explanation of the protocol is to get the reader familiar with the steps first, then point out the key points.

2.2 Is the PDMS secured to the device in any fashion? Are the electrodes reusable?
The PDMS layer is acting only as a seal and is fixed between device and an acrylic holder layer. Yes, the micro-calorimeter devices with electrical components are reusable.

2.3 It appears that the second sentence should be first on this step. Perhaps referring to the figure would help understanding.
The structure is changed and updated to clarify the section 2.3.

2.5.1 A viscosity range for accurate results would be useful.
The exact viscosity limit is not experimentally or theoretically determined, although the flow rate limitation (0.25 µL/min) is practically found during the experiments.

3.3 How is the acrylic layer introduced? Spin-coat?
The acrylic layer is a 5 µm double-sided adhesive film. We just need to place it on the top of the RTD device, as explained in section 3.3.

3.4 Please specify what solution or method to adjust the pH of the solution. Also, for those new to the method, please introduce GOD enzyme.
The pH of the buffer is controlled by the amount of acetic acid in the sodium acetate buffer. The note is added to lines 211-212 of the text.

3.6 Introduction to paper is via pipette.
The text is updated with suggested correction in line 229.

Line 316: Over what range can an experimenter expect the temperature linearity exist?
The temperature measurement linearity depends on the linear range of the RTD. For nickel, it is between 0-150 ^{o}C [3]. The linearity limitation for temperature measurement range is mentioned in the discussion section, line 344.

Fig 2. Label "beads" in diagram
Figure 2 is updated with suggested corrections.

Fig 3. In the description, "B)…" the author uses "the same bead" twice, seems like this is a minor grammar error.
The Figure legend is modified and updated.

Fig 4. Label "pins" and "device holder" in diagram for clarity.
Figure 4 is updated with the suggested descriptions.

Fig 6. Please describe and introduce the Keithley 2600 and 2400 in the body of the article.
The source/meters are used for measuring resistance of RTD sensor by applying constant current (Bias Current). At various points within the text, the function of the source/meters are explained (lines 135 and 223).Also, the reason for using two different types is mentioned in Figure 6 caption.

Fig 7. There are three data groups listed, but only two are in view on the graph.
In Figure 7B, “Given Data” and “Calorimetric Detection” are very close with minimal error and almost overlapping.

In the list of equipment amplifier is spelled wrong.
The list of materials and equipment are corrected and updated.

Reviewer #4:

Line 26: Replace 'micro-scale and nano-scale' with 'microfluidic devices'. There was no nano-scale data presented in the details of the three methods.

Suggestion is applied to the text.

Line 48: Consider changing 'Using heat' to 'using heat transfer'.

Suggestion is applied to the text.

Line 77: RTD was never spelled out. 'resistance temperature detector (RTD)'

RTD is defined as suggested.

Line 56: "compare to" should be "compared to"

Suggestion is applied to the text.

Line 82: Recommend adding the acronym TPD after Thermal Particle Detection since the acronym is used in this section.

Suggestion is applied to the text.

Line 86: Should add the words "processing technology as previously described in (2)."

This point is described in lines 89-90.

Line 88: The word fabrication should be removed and the sentence reworded, i.e. "To produce polydimethylsiloxane (PDMS) substrates with microchannels, a SU8 male mold needs to be created using standard lithography processes (5)"

The section 1.2 is reworded as suggested by the reviewer.

Line 100: This sentence is not clear. How is the manual punch "preparing the microchannel"? Please reword.

The section 1.3 is reworded and updated.

Section 1.4 - A figure of the actual device should be shown and referenced to in this section.

Figure 3 shows actual photograph of device.

Section 1.5 & 1.6 - Since this liquid is used for all 3 detection techniques, it is recommended that the preparation of the solution samples be listed as a separation section under Protocols, Section 1 Sample Preparation, then list Section 1.5 and 1.6 as 1.1 and 1.2, respectively. The TBD section should be listed as 2., Thermal Characterization of Liquid Substances using a Micro-Calorimeter as 3., and Calorimetric Bio-Chemical Detection in Paper-Based Microfluidic Device as 4.

Thank you for the suggestion. Since we are presenting thermal measurement and detection techniques in each section, we are explaining sample preparation for each method within the protocol. Introducing a separate section may add complexity and require further clarification for readers.

Line 109: Identify the acronym PS as polystyrene (PS).
Suggestion is applied to the text.

Line 111: Please reword this description to say, "To ensure the PS beads remain neutrally buoyant, 2.7 ul of glycerol must be added to DI water to match the fluid density to the PS bead density (1.05 g/cm3).
Section 1.6 is reworded as suggested by reviewer.

Line 114: How is the PTFE tube connected to prevent leaking?
Tight fitting made by selecting the right punch size will avoid leakage in tubes. The description is added to the text (Line 125).

Line 118: Recommend rewording to "…fill the whole channel with fluid all the way to the reservoir."
Section 1.8 is reworded, as suggested by reviewer (lines 128-129).

Line 120: If you add the "balanced" bead solution to the reservoir filled with DI water, won't this dilute the "balanced" bead solution, which may result in the beads settling out of solution? Please verify this will not be the case.
As explained in Section 1.8, the channel is filled all the way to the reservoir but not whole reservoir, so there will be slight dilution, which won't have any dramatic effect on buoyancy and the beads do not settle.

Lines 127-133: Recommend rewording to "…, the RTD sensor is electrically biased by applying a DC current in the range from 100 uA to 1 mA to continuously measure the temperature until the end of the counting experiment. It is critical to select the correct current level since there is a trade-off between noise level and the detected signal amplitude." The lines describing the flow rate vs speed of measurement and/or electrical measurement speed is not clear. For example, is there a particular method/protocol followed to "dial in" the counting signal? Please reword.
The mentioned section is reworded as suggested by reviewer. As explained in the following sentence, the speed of measurement is a function of the thermal time constant of the device and it will be affected by convective heat transfer through flow rate in microchannel.

Line 135: What is the "developed data processing software"? If described elsewhere in the manuscript, please reference.
It is a LabVIEW code, which converts the resistance to temperature based on the Callendar–Van Dusen equation, as explained in the text(lines 148-149).

Line 145: Suggest replacing the word "by" with "to"
Suggestion is applied to the text in line 160.

Lines 156-157: The computer controlled program is mentioned a few times. Is this program in LabVIEW or some other platform? What specific parameters must be specified for the computer-controlled program?
As explained earlier, it is a simple LabVIEW code to converts the resistance to temperature.

Line 159: Is there a flow profile programed? Steps, pulses, or ramps?
The flow profile is constant and a pump is used in Push Bottom mode, and the term “constant flow” is added to section 2.5.1.

Line 167-170: How is heat penetration time measured? Is it by evaluating temperature vs time of a heat pulse, if so please state the protocol/methods used to determine this parameter. Recommend clarifying in Figure 5A which Keithley 2400 is used to supply power to the heating element and which is used to measure the resistance of the RTD.
The circuitry for measuring the heat penetration time is presented Figure 5(A). The temperature penetration time is the time difference between applying heat pulse to the heater and detecting modulated pattern at the sensor. The Figure 5(A) is updated for more clarification.

Line 200: Please define acronym GOD, i.e. Glucose Oxidase (GOD) enzyme.
The GOD is described in the first use within the manuscript in line 73.

Line 202: Should specify how the pH of the solution was adjusted to 5.1?
A description is added in lines 219-220.

Line 211: Please expound as to why the "detected temperature must start to decrease"
Sections 3.6 & 3.7:
The temperature decrease is explained in lines 232-233 in the text.

Section 3.7 appears to be the calibration of the system with different glucose concentrations, if so, shouldn't the calibration step come before the actual prepared sample measurement step?
Actually, we used different calibrated samples to measure our device accuracy, not to calibrate our device. So we used the well-known control solutions to validate our measured values in this experiment.

Line 219: Should the word resistance "date" be "data"?
Mentioned typo is corrected to the text.

Line 231: Recommend rewording "optical corresponding images" to "corresponding optical images"
Suggestion is applied to the text.

Lines 238-245: It is unclear whether particle size or particle location relative to the heat source and detector is being determined. Are the authors saying that the larger particle drops the resistance while

the smaller particle increases the RTD resistance? How do they distinguish the effect of particle location within the channel verses particle size? If the smaller particle was closer to the bottom electrodes would the RTD resistance still increase, or would it decrease like the larger particle? Is there a particle size between the small and large particle that would produce no resistance change? Fluid flow will directly effect thermal conductivity of the RTD, how are the authors able to clearly distinguish between the effects of the particles and fluid flow on thermal conductivity? This paragraph should be expanded to provide more detail on the actual detection system, which will hopefully clarify these questions. Also, figure 5 shows the device in thermal isolation, but this was not adequately described in the text since the ambient temperature would also have an effect on the system. Please add a description and discuss.
The heat transfer in the microchannel is direct function of the flow rate (lines 142-146). However, the thermal conductivity (effective thermal conductivity) of the channel (beads + medium) is affecting the heat transfer in the channel. As described in the results section, in the case of the smaller bead size to the channel size ration the thermal conductivity will be a dominant phenomenon to affect the heat conductivity (lines 249-255). By increasing the bead size to channel size ration, the local fluid velocity change will be dominantly affecting phenomena (lines 257-264). The effect of the ambient temperature isolation is mentioned in the discussion section (lines 333-335) and demonstrated in the experimental setup Figures.

Lines 247-250: What is the significance of the data in table 1? The nomenclature for the Samples is not relevant to the reader. Please define the differences between the types of samples listed in the table.
The Table data is republished with permission from prior publisher.

Line 274: The word "dimensional" in 3-dimentional is misspelled.
The correction is applied and can be found in line 293.

Line 279: Has the acronym TWA been defined?
Yes, The Thermal Wave Analysis (TWA) is defined in its first use within the article in lines 59-60.

Discussion section: The limitations and problems associated with the technique presented are not adequately discussed in this section. Please expand.
The required thermal isolation from ambient temperature fluctuations (lines 335-337) and the RTD sensors linear measurement limit (line 344) are added to the discussion part. The thermal time constant design criteria are explained in the discussion section (lines 329-335). More details on heat transfer parameters that were taken into account in design and fabrication of the on-chip calorimeter device were explained in a prior publication and cited in references [3].

Figure 2: This schematic does not provide a lot of insight given the nature of this journal. An image of the actual experimental setup would be more useful and this figure only shows one Keithley meter, is this correct? Only one Keithley meter is used during the experiment? The description above suggests at least 2 meters are used during the experiment.

Yes, only one source/meter (Keithley) is used in thermal particle detection. A single RTD is used in this experiment as a heat source (heater) and temperature sensor as indicated in lines 138-142. The bias current of RTD sensor is used as the input power (heat source) in this experiment.

Figure 5: How is the temperature from the heater shown in Figure 5 A and Figure 5 B monitored? How much power is being supplied by the heater?

Our device uses RTD to measure the temperature, as indicated in line 158. For heat penetration measurement DC current is being applied to the heater, however, the alternating voltage is being applied for specific heat measurement. The details of the measurements are addressed in reference [3].

Figure 6: All other figures state a Keithley 2400 meter was used, yet this figure indicates a Keithley 2600 was used. Is this correct?

Yes, it is correct. In the first two experiments (thermal particle detection and micro calorimetry), we used Keithley 2400. To measure the data faster in the paper-based calorimetric microfluidic device, we used Keithley 2600. The measurement speed is mentioned in the Figure caption.

Figure 7b: The word "calrotimetric" is misspelled. Also, what is "Given Data" please define.

The misspelling is corrected in the Figure 7B legend and the “Given Data” is defined in the Figure caption, as suggested by reviewer.

Table 1: Is the data in the third row of Table 1 correct? This value seems like an outlier. As a comparison, it would be useful to know what the theoretical or standard values are.

Yes, values are correctly listed in the table. The Table data is republished with permission from prior publication.

In the materials and equipment list, Sylgard and Glucose are misspelled.

The Materials and Equipment list are updated with the corrections.

Figure 3 is not referenced before figure 4 in the document.

Figure 3 is addressed before the Figure 4 on lines 146.

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