**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.

6. JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.  
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 oC [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.