

Measurement of Temperature and pH Value in Microplates Using Two-Colour Laser-Induced Fluorescence Spectroscopy

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ABSTRACT

The two-colour laser-induced fluorescence (2c-LIF) technique enables the simultaneous spatial and temporal resolution of temperature and pH fields in liquid systems. In view of the continuous growth of the life sciences sector, particularly in molecular biology and diagnostics, this method offers significant potential for improving analytical and screening processes under realistic operating conditions. Within temperature-controlled microplate wells, confined liquid volumes in combination with vertical temperature gradients lead to the formation of thermally induced flow structures. These flows strongly influence scalar transport and mixing behaviour, which are critical for the performance and reproducibility of microscale assays. A detailed investigation of these coupled transport processes is therefore essential.

In this study, a Fluorescein/Sulforhodamine 101 dye system in aqueous solution is used for parallel spectroscopic and imaging-based thermometry in the temperature range from 283 K to 343 K. The spectroscopic approach achieves temperature sensitivities between 2%/K and 6%/K, while the imaging-based method provides sensitivities from 2%/K to 3.5%/K. This corresponds to a statistical temperature precision of approximately 0.23 K to 0.50 K. Absorption-corrected dual-camera imaging enables quantitative full-field temperature measurements and allows the temporal evolution of thermally driven flow structures to be resolved during dynamic heating processes. These thermal movements are quantified using the local Peclet number. In buffered solutions (pH 6–11), the fluorescence intensity ratio exhibits a linear response in the range between pH 6 and 9, enabling spatially resolved visualization of biochemical mixing. To quantify mixing dynamics, time-resolved isothermal droplet injection experiments are conducted in the absence of external forcing. A normalized mixing index based on the coefficient of variation is introduced to determine homogenization times and to assess the relative contributions of advective and diffusive transport. Overall, 2c-LIF provides a robust diagnostic framework for investigating coupled heat and mass transfer in confined microplate systems and for analysing transport-driven mixing processes in microscale liquid volumes.

1. Introduction

The automation of laboratory processes is an essential step towards achieving a higher throughput while maintaining consistent quality. Various industrial sectors, such as pharmacology,

biotechnology, chemistry, and biomedical technology, benefit from these developments. A key component of automated liquid-handling platforms are devices designed for precise heating, cooling, and mixing of fluid samples. Many of these processes take place in microplates or tubes. This allows a large number of samples – typically 96, 384 or 1536 – to be analysed under identical conditions. This is used, for example, in medicine for virus detection (Huang et al., 2021), in research into new vaccines (Harbison-Price et al., 2025) or in research on cancer cells (Widder et al., 2018). New yeast cultures for the production of medicines (Hebra et al., 2024) or for the food industry (Zhao et al., 2022) are also grown in microplates. Similarly, many food analysis processes are carried out in such microplates (Jung et al., 2023; Osiecka et al., 2024). To further improve process quality and throughput, a detailed understanding of the quantities within these devices is required. Among the most relevant quantities are the temperature, pH value, and solute concentrations in the sample fluid.

To determine the process quantities with high spatial and temporal resolution, optical methods offer clear advantages. They provide fast, contactless, and non-invasive measurements, ensuring minimal disturbance of the sample while maintaining high accuracy and providing spatial resolution. In this study, two-colour laser-induced fluorescence (2c-LIF) spectroscopy was applied to measure thermodynamic quantities in aqueous dye solutions contained in microplates. This technique is frequently used to measure thermodynamic parameters in liquids or multiphase systems. Examples of its application in research include temperature measurement in liquid films (Koegl et al., 2024), sprays (Mishra et al., 2016) and droplets (Ulrich et al., 2024). A mixture of Fluorescein sodium salt (FL, CAS: 518-47-8) and Sulforhodamine 101 (SRh, CAS: 60311-026) was applied as a dual-dye system, combining a temperature- and pH-sensitive fluorophore with an insensitive reference dye. This dye combination was originally proposed by (Chaze et al., 2017) for studying liquid jet temperature and later successfully applied by (Ulrich et al., 2024) in sprays and droplets.

2. Experimental setup and materials

The experimental setup used is shown schematically in the Figure 1a and can be divided into three separate subsystems. The exact model numbers of the devices used, along with the manufacturers and specifications, are summarised in Table 1. In the first section, the continuous-wave laser is shaped into a laser sheet using two cylindrical lenses. A subsequent aperture is used to further shape the beam. The laser sheet is evenly split at a beam splitter. One beam enters the second part of the setup, which is intended for analysing the laser sheet. This section includes a power meter

to measure the laser power and maintain it constant throughout multiple measurements. A beam profile camera is also integrated. This camera is used to analyse the beam profile in order to make subsequent corrections to the excitation laser. The second part of the laser sheet enters the third section of the setup, where the actual sample analysis takes place. The sample and reference vessels are heated in a controlled manner on an industrial heating device. The fluorescence light is analysed in two ways. First, using a fibre-coupled spectrometer that collects the fluorescence light via a fibre-coupler. Second, using a camera system that collects the fluorescence light via a long-distance (LD) microscope objective. A notch filter absorbs the excitation light in both setups: for the spectrometer setup before the fibre-coupler and for the camera setup between the LD microscope objective and a dichroic mirror. The notch filters have a central wavelength (CWL) of 533 nm. In the camera setup, after passing through the microscope objective, the fluorescent light is split by a the dichroic mirror. The CWL of this mirror is 588 nm. The two beams are then detected by two sCMOS cameras. Both cameras are equipped with bandpass filters to analyse the different wavelength ranges. The “green” channel is detected using a bandpass filter with a central wavelength of 554 nm (554/23 BrightLine HC). The “red” channel is detected using a bandpass filter with a central wavelength of 615 nm (615/24 BrightLine HC).

Individual microplate wells are used as sample vessels (Figure 1b). These are cut from 96-well plates and are made of polystyrene. The wells have a flat bottom (F-bottom) and a maximum filling volume of approximately 350 μl . Typical working volumes in this study are approximately 200 μl . To ensure that the thermal conditions of the measurement reflect those in reality, insulation panels made of epoxy resin are placed around the individual vessels. These panels reduce heat loss through convection, thermal conduction and radiation, whilst still allowing the measurements to be carried out. Thermocouples are used to provide a second method of temperature measurement in the reference vessels. Type T thermocouples with a measuring range of 233 K to 398 K are used for this purpose. The sheath diameter is 0.5 mm. The data is recorded using a USB thermocouple logger. A spectrophotometer with a temperature-controlled sample holder is used to determine the absorption coefficient of the dye solutions. Quartz glass cuvettes (10 mm $\times$ 10 mm) are used as sample vessels for this purpose.

Solutions of distilled water and the two dyes FL (50 mg/l) and SRh (5 mg/l) are used as the sample liquid. For the pH-dependent measurements, which were also carried out, aqueous buffer solutions with a pH value between 6 and 9 are used. The same dye concentrations were used for all buffer solutions as for distilled water.

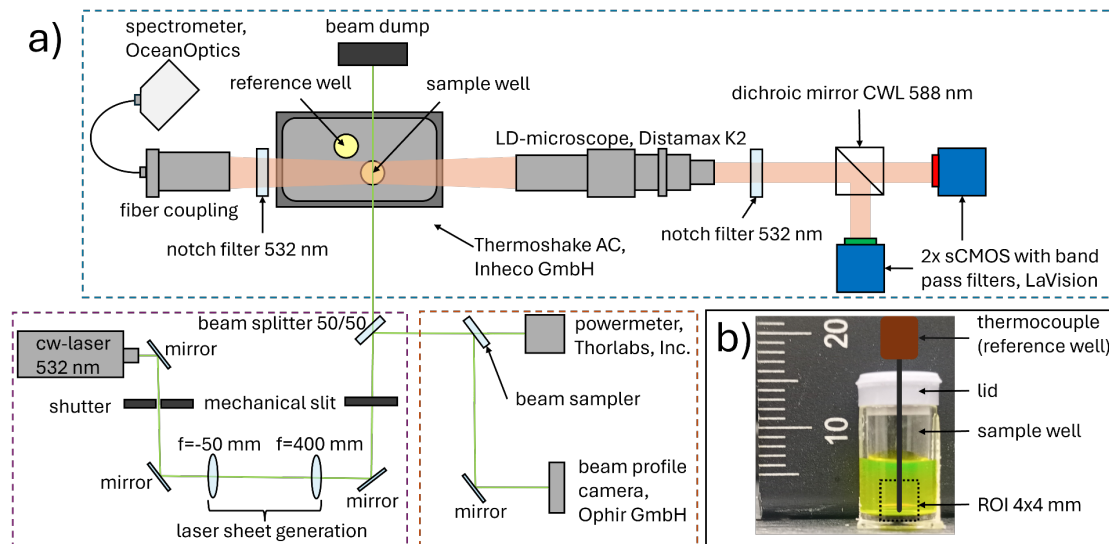


Figure 1 a) Schematic diagram of the experimental setup, comprising the laser-sheet optics (outlined in purple), the beam diagnostics (outlined in orange) and the sample measurement using a spectrometer and camera system (outlined in blue). b) Sample vessel filled with dye solution. Also shown schematically are the position of the thermocouple in the reference vessels and the ROI of the camera system.

Table 1 Overview of the equipment used in the experimental setup, including manufacturer details and specifications.

Components	Manufacturer	Model	Specifications
Nd:YAG cw-laser	Roithner, Austria	RLTMGL-532	Wavelength: 532 nm Power: 2.5 W Stability: <5 %
Spectrometer	OceanOptics, Germany	HR4	Wavelength range: 400 - 850 nm Slit size: 10 μ m Optical resolution: 0.48 nm
Powermeter	Thorlabs, USA	S121C	Resolution: 10 nW Uncertainty: ± 3 %
Beam profile camera	Ophir, Germany	L11059	Resolution: 4008 px $\times$ 2672 px Spectral range: 190 - 1100 nm
sCMOS camera	LaVision, Germany	Imager CLHS	Resolution: 2560 px $\times$ 2160 px Readout noise: < 3 e ⁻
LD-microscope	Infinity, USA	Distamax K2	Resolution: 81 px / mm
Spectrophotometer	Jasco, Germany	V-750	Wavelength range: 190 - 900 nm Accuracy: ± 0.2 nm
TC-logger	Pico Technology, UK	USB TC-08	Accuracy: ± 0.2 %

3. Results

In a first measurement series to characterize the result of the technique, the sample was heated from a temperature of 283 K to 338 K in 5 K steps. The spectral and imaging measurements were carried out simultaneously at a laser power of 10 mW. In each case, 50 spectra and 50 images were recorded at a frequency of 1 Hz. Both the images and the spectra were subsequently averaged. Figure 2 (left) shows one such averaged spectra. The temperature dependence of FL can be seen in the area marked in green. SRh, on the other hand, shows hardly any change with temperature in the area marked in red. The normalised ratios of the two channels show an increase with rising temperature, which can be described by a third-order fit function (Figure 2, right). The derivative of this function with respect to temperature gives the sensitivity of this function. The temperature sensitivity was found to lie between 2 %/K and 6 %/K.

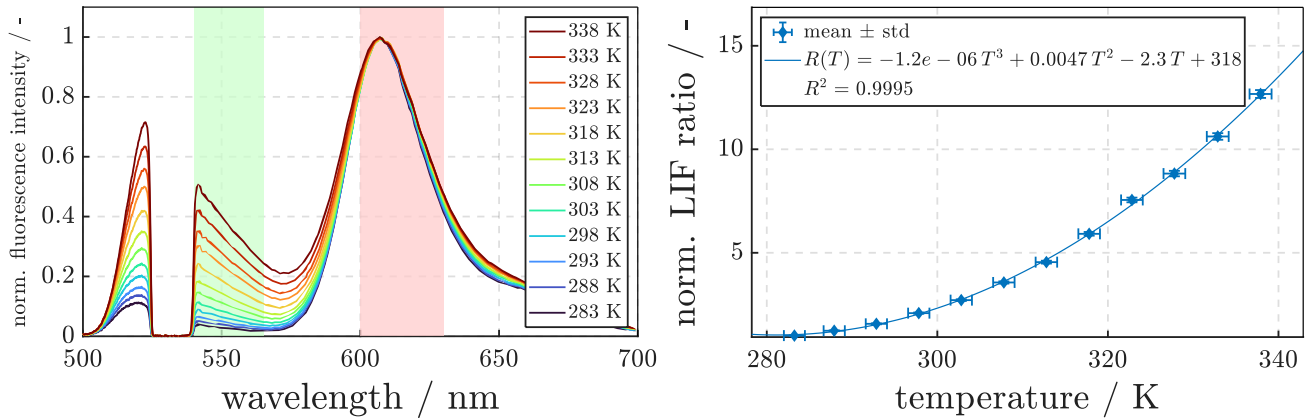


Figure 2 Left: Spectrum of normalised fluorescence intensities in the temperature range from 283 K to 338 K. Right: LIF ratios normalized to the lowest temperature of 283 K.

For the imaging measurement, the ratio of the two camera images was calculated. The region of interest (ROI) under consideration measures 4.98 mm by 4.71 mm. To align the images precisely, a target image was first captured and superimposed onto these images using an algorithm. The calculated deviations were then applied to the 2c-LIF images. The calculated LIF ratios are shown in the Figure 3. These points can also be fitted well using a third-degree fitting function. The calibration function is shown in the figure. The temperature sensitivity for the camera images was found to be between 2 %/K and 3.5 %/K. This calibration function allows to convert the captured images into temperature fields. To do this, the LIF ratio was calculated pixel by pixel from the image files and then converted into a temperature. The temperature fields for each set temperature are shown in Figure 4.

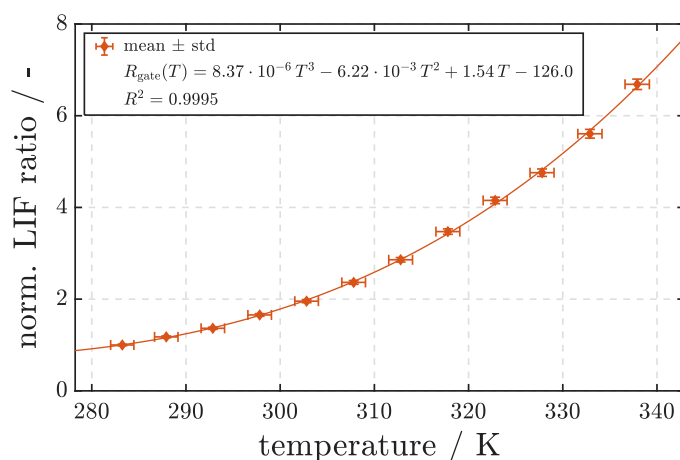


Figure 3 LIF ratios normalized to the lowest temperature of 283K.

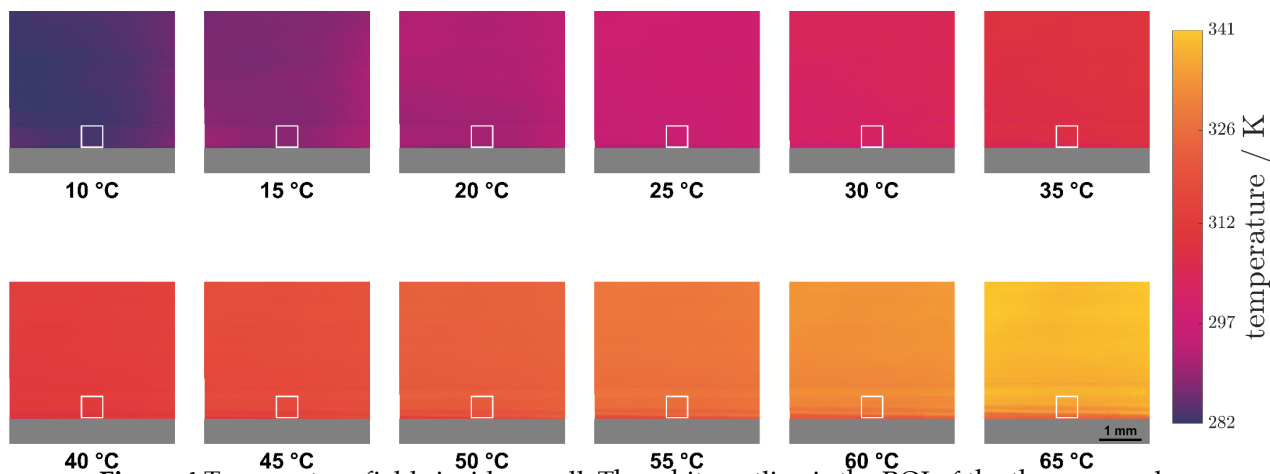


Figure 4 Temperature fields inside a well. The white outline is the ROI of the thermocouple.

Reference values for sensitivity are found in literature, for example in the work of (Sutton et al., 2008) and (Estrada-Pérez et al., 2011). They investigated similar dye pairs in aqueous solutions. A comparison of these literature values and the sensitivities measured in this study is shown in Figure 5.

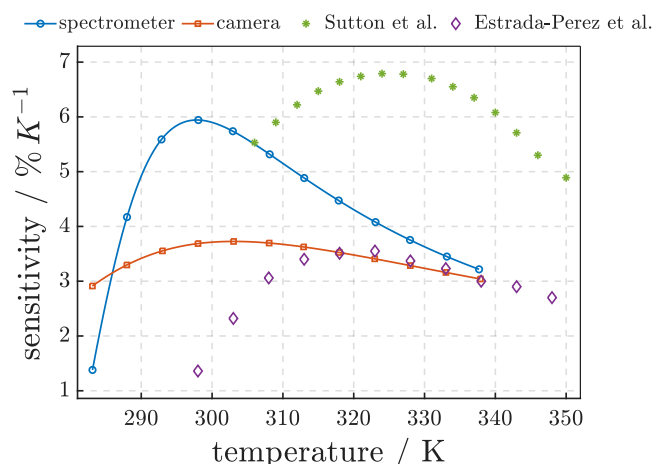


Figure 5 Comparison of the measured temperature sensitivity for the spectrometer (blue circle) and the measured sensitivity for the camera (orange square). For comparison the sensitivities measured by Sutton et al., 2008 (green asterisk) and Estrada-Pérez et al., 2011 (purple diamond).

Various influencing factors were considered to estimate the measurement error. For the spectrometer measurements, both the repeatability of the measurement ($\sigma_{SM,stat} = 0.5\%$) and the instrument error of the spectrometer and the optics ($\sigma_{SM,sys} = 1.41\%$) were considered. This results in a total error of 1.5%. This corresponds to a temperature deviation of 0.23 K to 0.50 K. For the imaging camera measurements, the repeatability of the measurement was also considered ($\sigma_{CM,stat} = 0.07\%$). In addition, the images were corrected to compensate for the influence of dye absorption between one end and the other end of the ROI. To this end, the temperature-dependent absorption coefficients of the two dyes were determined using a spectrophotometer. The correction was then carried out using Lambert-Beer's law. The associated error was determined to be $\sigma_{CM,ab} = 1.33\%$. Finally, the error arising from fluctuations in the beam profile was considered ($\sigma_{CM,bp} = 1.04\%$). The total error is 1.7%. This results in a temperature uncertainty of 0.41 K to 0.57 K. In addition to temperature measurements, pH measurements were also carried out. Here, the pH-dependence of fluorescein was utilised (Lacassagne et al., 2018). In the pH range from 6 to 9, a linear relationship between the pH value and the LIF ratio was observed. The calibration curve is shown in Figure 6.

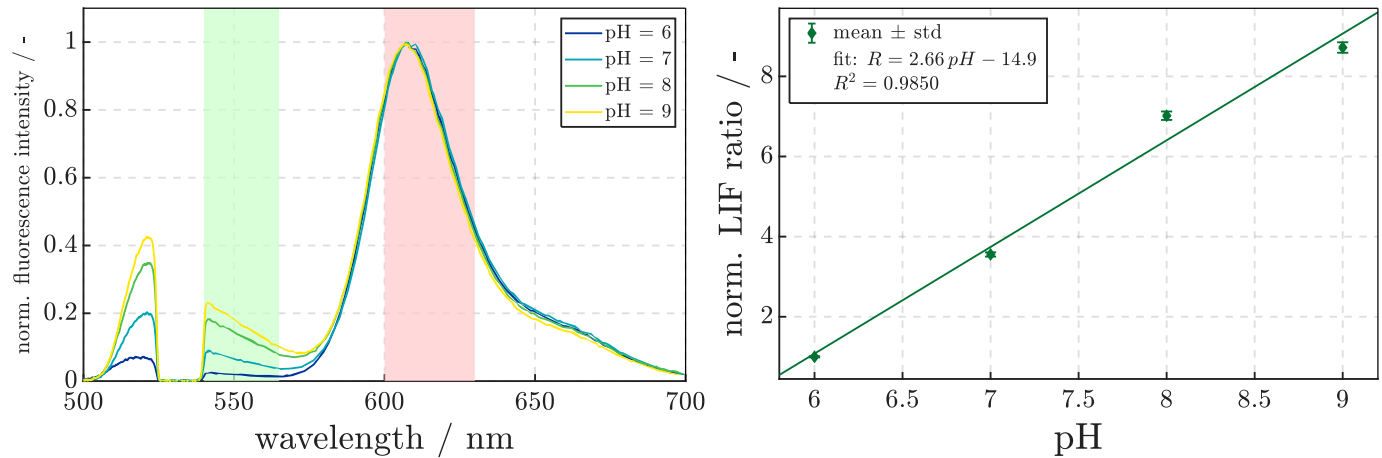


Figure 6 Left: Normalized fluorescence spectra of different dye buffer solutions in the pH range of 6 to 9 (at 293 K). Right: Normalized LIF ratios and linear fit function.

This relationship between pH value and fluorescence intensity was used to investigate the mixing of two liquids. To do this, a buffer solution (pH=9) was pipetted into a microplate well filled with another buffer solution (pH=6). The sample volume was set to $200\mu\text{l}$ and the droplet size was varied between $10\mu\text{l}$ and $50\mu\text{l}$. The measurement was carried out at 293 K without any external forces such as shaking. The recordings were run at 5 fps. The coefficient of variation (*CoV*) was calculated as a measure of homogeneity. This *CoV* is the ratio of the standard deviation σ of the signal to the mean value μ of the signal.

$$CoV = \frac{\sigma}{\mu} \quad (1)$$

To achieve better comparability of the curves, they are normalized to the range between the initial baseline CoV_{base} and the maximum deflection CoV_{peak} . This mixing index $M(t)$ is defined as:

$$M(t) = 1 - \frac{CoV - CoV_{base}}{CoV_{peak} - CoV_{base}} \quad (2)$$

The mixing time t_{95} should be the time it takes for $M(t)$ to return to above 95% of its initial value. An overview of the mixing of the droplets in the vessels can be seen in the Figure 7 left. This shows the mixtures at various points in time after the start. The mixing index for all droplet sizes is shown in the Figure 7 right. As expected, it can be seen that as the droplet size increases, it takes longer for the two liquids to mix.

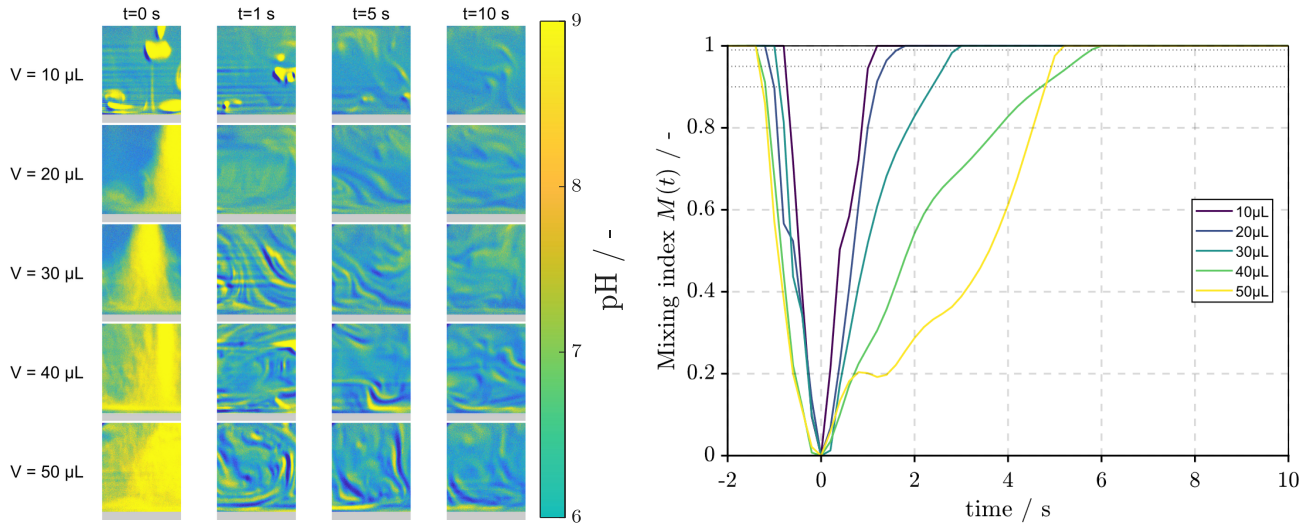


Figure 7 Left: LIF images for the two-fluid mixture at different drop sizes and times. Right: Mixing index for different drop sizes.

4. Conclusions and Outlook

As part of this work, a measurement setup was designed to measure the temperature profile and thermal movements in micro-plates. Using the dye pair of Fluorescein sodium salt and Sulforhodamine 101, sensitivities of between 2%/K and 6%/K were achieved for spectrometer measurements and between 2%/K and 3.5%/K for camera measurements. The highest sensitivity was measured within a temperature range of 283 K to 308 K. This temperature range is important for many applications in biochemistry and biomedicine. Sensitive measurement within this range is therefore advantageous.

Furthermore, the pH-dependent LIF signal enables observation of the mixing of two fluids inside a micro-plate well. This made it possible to determine the mixing times when pipetting different droplet sizes. This provides insight into how well mixtures homogenise and how long this process takes.

As a next step, other liquids that are also important for use in microplates will be investigated. Examples include dimethyl sulfoxide, propanediol and various alcohols. To do this, first, a suitable dye combination must be identified, followed by the calibration of the adapted setup.

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