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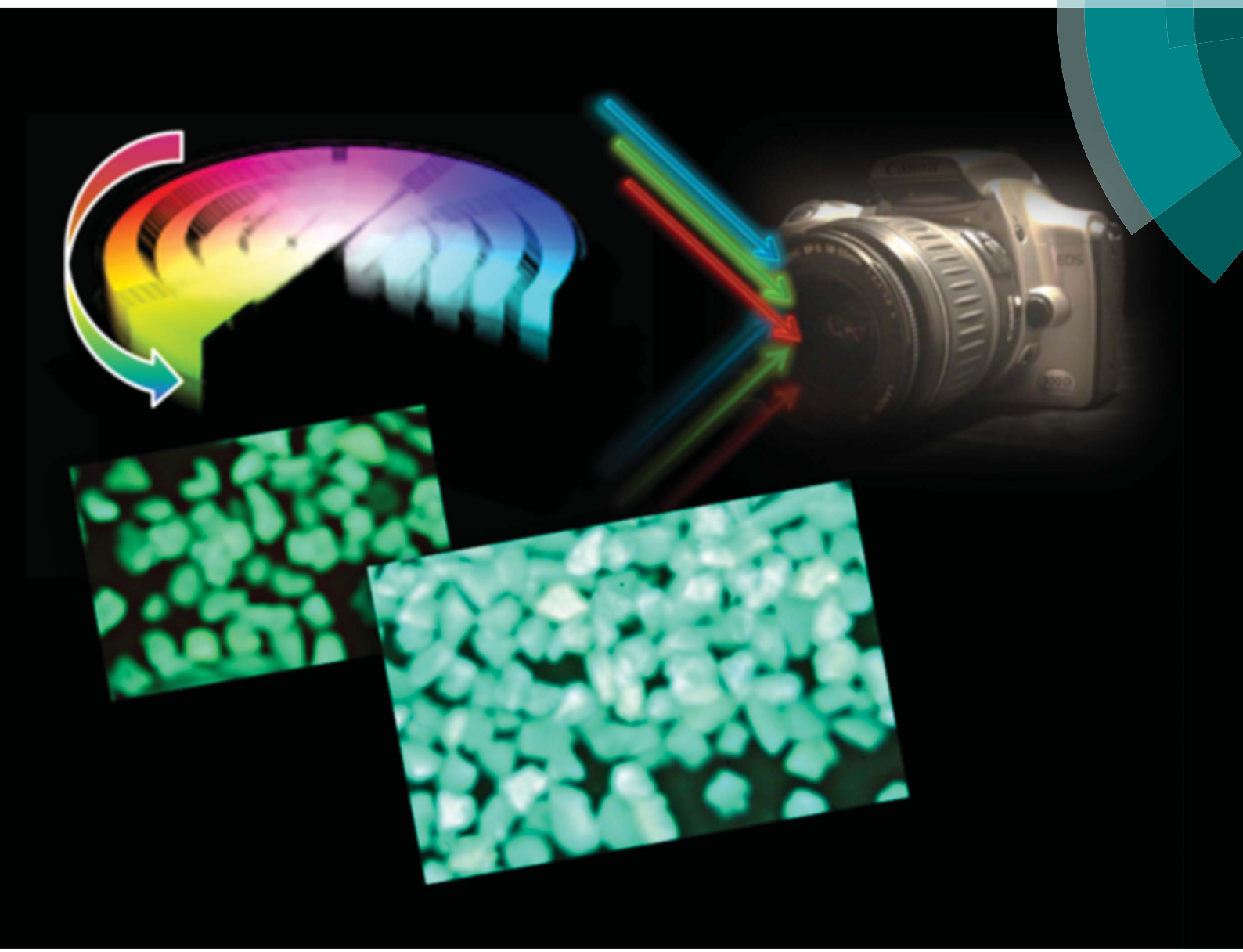

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The hue parameter of HSV colour-space for digital imaging is shown to be accessible for convenient quantitative fluorescence imaging. A commercially available pH probe was utilized in solution and incorporated into optical μ-sensors for microscopy applications.

Optical chemical sensors including plasmonic and nanotechnology approaches are rapidly becoming established as efficient tools for high-resolution sensing and imaging of chemical distributions.[1–9] Modern digital colour cameras are cheap and excellent in performance, offering opportunities for optical chemical measurements with improved simplicity, cost and performance. Colour sensitive digital imaging devices such as CCDs and CMOS sensors provide a convenient real-time three part RGB-signal in each pixel. The use of these three colour channels of digital cameras, in combination with cheap and robust LED light sources, can reduce or even eliminate the use of expensive and fragile optical filters and filter wheels and allow direct reading of wavelength relevant information from each pixel.

HSV (Hue, Saturation, Value) is a commonly used cylindrical colour-space in digital imaging. Hue (H) corresponds to the CCD "spectral" colour tone represented as angular values from 0 to 1 (circular part of the cylinder). Saturation (S) represents the maximum difference between the RGB channels (cylinder radius) and value (V), sometimes referred to as brightness, B, is the maximum channel value (cylinder height). The equations for HSV colour-space:

$$H = ((G - B)/(\max - \min) + 0)/6; \text{ if } R = \max$$

$$H = ((B - R)/(\max - \min) + 2)/6; \text{ if } G = \max$$

$$H = ((R - G)/(\max - \min) + 4)/6; \text{ if } B = \max$$

$$S = (\max - \min)/\max$$

$$V = \max$$

The fluorescent pH sensitive dye di-hydroxypyrene-di-sulphonate (DHPDS, Sigma Aldrich) displays a convenient blue to green shift with increased pH when excited at 405 nm.[10,11] Dye dissolved fluorescence images of DHPDS in different pH buffers (Merck, NIST) and different modes are shown in Fig. 1. Images were taken with an inexpensive Fujifilm Finepix XP30 compact

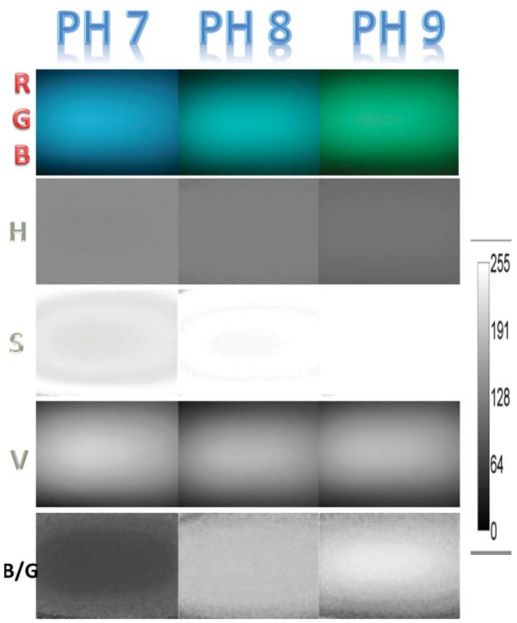

Fig. 1 Different modes of pH dependent images (pH 7.00–9.00, left to right column). RGB colour, hue (H), saturation (S), value (V) and blue/green ratio (B/G) image modes from top to bottom row. Scale bar does not apply to the RGB images (top row).

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camera (~$200), and the excitation light-source was a 405 nm LED device made in-house (~$20). Image assessments were made in National Institute of Health's open access software ImageJ.[12] Fig. 1 shows uneven distributions in most modes, even in the ratiometric mode. However, the uneven distribution was almost completely eliminated in the hue images (Fig. 1). The difference is most clearly seen in the corners and edges when compared to the second best mode for homogeneity (*B*/*G* ratiometry). The saturation and value images were unsuitable for direct quantification. However, they can likely provide supportive qualitative information in each pixel that can be used to remove inconsistent data and outliers in a convenient and automated manner. For example, low saturation may indicate high background or loss of signal dynamics. For extremely demanding and high-performance applications, active seeking of *S* and *V* values in specific intervals, using automated scripts in *e.g.* Matlab or ImageJ, may be an approach for accuracy improvement and quality validation.

The hue quantification of dissolved DHPDS fluorescence demonstrated a precision of 0.001 pH units (IUPAC pooled standard deviation, pH 7, 8 and 9, $n_{tot} = 18$, example images in Fig. 1), which is close to the performance limit for samples under indoor ambient conditions (mainly due to CO_2 perturbation).[11] The precision was approximately a factor of three better than ratiometric measurements calculated from the same data. The negatively charged DHPDS was further swiftly immobilized on quaternary ammonium functionalized ion exchange microparticles (Sigma Aldrich, for HPLC; 25–30 μm particle size). 100 mg particles were put into 2 ml 10 μM DHPDS and shaken for 5 min. Then the solution was centrifuged, the supernatant was removed and the particles were washed with MilliQ water and dried at 50 °C.

Microscopy images of the particles at pH 6 and 7 are shown in Fig. 2. The significant color difference between dissolved (Fig. 1) and immobilized (Fig. 2) DHPDS is the consequence of both the immobilization process[13] and the use of two entirely different imaging systems. Also, a small number of particles at pH 6 were overexposed (~10 very bright particles), however these particles also showed clearly deviating *S* and *V* values, and inconsistent pH values can easily be picked out.

With hue-parameter normalization, the particle by particle precision defined as pooled standard deviation was 0.003 pH units (6 replicates, 6 particles, pH 6 and 7, $n_{tot} = 72$), which is similar to some of the best performing immobilized dye based optical sensors for macroscopic use, for example see ref. 13 and 14.

These particles can conveniently be used in any microscopy application where high-precision pH control or subtle monitoring is important. For example, during *in vitro* fertilization pH is a key factor for the success rate,[15] and isolated yet close high-precision microscopy pH measurements can be indispensable. The only hardware needed are a colour camera and a Leica A filtercube (or similar, dichroic mirror @ 400 nm and LP 425), commonly present in fluorescence microscopy set-ups.

To further assess hue fluorescence quantification and directly compare the applicability of hue *versus* ratiometric and intensity based sensing, a CMOS color camera (Thorlabs |Model| 1645C) was used as a macroscopic fluorescence detector. The set-up and experimental design were the same as in the study of Hakonen *et al.*,[11] however with no long-pass filter (or any filter). The excitation light source was a 405 nm Thorlabs mounted LED at minimum power and dissolved DHPDS (3 μM) mixed with buffers continuously in a 25 μl flow-cell (flow-rate 334 μl min^{-1}, one measurement every 15 s.). Calibration data (averaged over entire CMOS) are shown in Fig. 3, and they

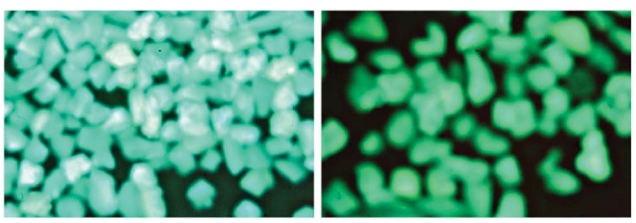

Fig. 2 Microscopy (Leica) images of 25 μm sized pH-optodes at pH 6 (left) and 7. Leica filtercube A (sufficiently close to optimum 405 nm excitation) was used. Images were acquired with a Canon 450D camera.

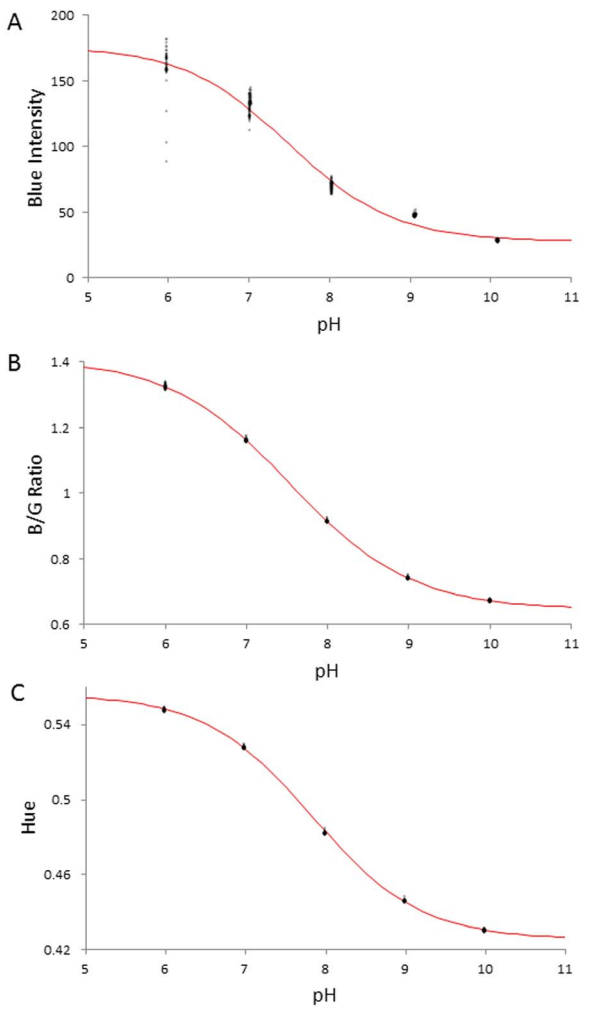

Fig. 3 Calibration curves for blue intensity (A), blue/green ratio (B) and hue (C) fluorescence data from 448 images (Thorlabs 1645C).

followed a smooth four parameter sigmoidal fit in all three modes.[13] A huge advantage for hue and ratiometric data over intensity based data can clearly be seen in Fig. 3, while discrimination between the two normalized datasets is less obvious. To evaluate the accuracy (mean error) and precision (pooled standard deviation), 100 pH determination experiments at pH 8.00 and 9.00 were performed (Fig. 4), demonstrating the best performance for the hue based quantification with an accuracy of 0.016 and a precision of 0.002 pH units. Ratiometric sensing showed an accuracy of 0.024 and a precision of 0.003 pH units and the intensity data displayed poor performance with an accuracy of 0.303 and a precision of 0.037 pH units.

Previously absorbance based optical chemical sensors using hue quantification have been demonstrated.[16,17] However, to the best of our knowledge there are currently no reports of fluorescence based hue chemical quantification and imaging. Although, similar but more complex principles without any chemical quantification were very recently (this month) accepted in the RSC journal *Chemical Science* and highlighted by Wiley's *Chemistry Views*.[18]

To use fluorescence detection rather than absorbance for hue quantification is a more challenging task, with additional demands such as excitation light removal and bi- or tri-tonal emission light with single wavelength excitation. Also, to perform quantitative fluorescence in high-resolution imaging extends the challenge even further. Fluorescence detection has significant advantages over absorption, and provides a scope for remarkable improvements in sensitivity, dynamic range and versatility. A fluorescent probe can effectively be utilized for *H*-parameter quantification if it displays a significant colour change (bi- or tri-tonal), across the borders of the RGB sensitivity (R → G; G → R) of the imaging device used. The most likely optimal fluorophores for hue normalization have a red shift in emission response from the intercept of blue-green sensitivity (blue side) to the green-red intercept (red side), both of which are device dependent but usually at approximately 500 and 580 nm, respectively. This would involve all three channels in a major way for maximum sensitivity.

A sensing scheme that likely is appropriate for *H*-parameter assessment is a recently developed nanoparticle enhanced/quenched sensing scheme.[19] Subsequently that sensor has been used for ratiometric imaging of ammonium and ammonia release from biological tissues, demonstrating unprecedented performance and capabilities for easy transformation into any cation sensor.[19–22] The dual excitation and dual emission sensing scheme originally employed for the sensor without plasmonic nanoparticles is not feasible for hue measurements.[23] However, as recently shown, a single excitation dual emission relaxation path is realized for the nanoparticle enhanced sensor.[24] The nanoparticle induced fluorescence ratio displayed an isosbestic point at 581.4 ± 0.1 nm, which is close to being ideal (G → R shift) for hue employment with most RGB imaging CCD and CMOS devices.

Another sensing scheme with potential for hue quantification may be from a recent paper where the authors used fluorescent thioxanthone dyes in different organic solvents to generate colours ranging from deep blue to deep red.[25] For such solvent sensitive dyes the *H*-parameter may be conveniently used to unveil complex mixtures of organic samples for on-line processes in a quantitative or at least a semi-quantitative way to complement other more costly and complicated off-line analyses.

In conclusion, hue quantification is demonstrated to be superior to ratiometric and intensity based methods by a factor of >1.5 and >15, respectively, and may be a powerful complement to time-dependent calibrations for further improvement.[13,23] Also, by using HSV for quantification, maximum utilization of the benefits of imaging devices can be achieved by robust extraction of "spectral" and signal quality information in each pixel. Design of novel and device matching fluorophores and sensing schemes (toward specific LED and CCD or CMOS used) can likely revolutionize further development in this direction. This will open up a new field of low cost analytical devices yet with excellent performance for tomorrow's increasing demands.

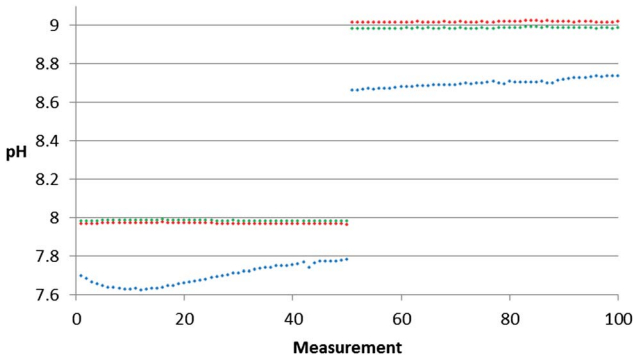

Fig. 4 Calculated pH of NIST buffers (Merck, pH 8.00 and 9.00) for comparison between intensity (blue), ratiometric (red) and hue (green) based fluorescence quantification (calibrations from Fig. 3).

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