

Electronic Supplementary Information

Expanded Single-Color Barcoding in Microspheres with Fluorescence  
Anisotropy for Multiplexed Biochemical Detection

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### Derivation of theoretical FA-based encoding number in a single color channel

The apparent FA' value obtained from FAIM is calculated by eqn (S1),

$$FA' = \frac{I_{VV} - I_{VH}}{I_{VV} + 2I_{VH}} \quad (S1)$$

where  $I_{VV}$  and  $I_{VH}$  are measured greyscale intensity values of each pixel from the channels VV and VH in FAIM (shown in Fig. S1).

By definition, the actual FA value is expressed in eqn (S2),

$$FA = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (S2)$$

where  $I_{\parallel}$  and  $I_{\perp}$  are true intensity values with the relevant orientations of emission polarization.

As shown in eqn (S3), the measured intensity ratio of FAIM is varied from the actual value by the instrument-dependent polarization sensitivity factor  $G$ , the ratio of sensitivities of the detection system for vertically and horizontally polarized light.

$$\frac{I_{VV}}{I_{VH}} = G \frac{I_{\parallel}}{I_{\perp}} \quad (S3)$$

Combining the above eqns gives eqn (S4),

$$FA = \frac{1 + G(FA' - 1) + 2FA'}{1 - 2G(FA' - 1) + 2FA'} \quad (S4)$$

For PS microspheres doped with 0.2% w/w DPH, the average FA' value from FAIM was 0.459, with the standard deviation ( $\sigma'$ ) equals to 0.006. Measurement of PS suspension in spectrophotometer FLS1000 gives the true FA value of 0.218. Therefore, from eqn (S4), the calculated  $G$  value of FAIM in the emission range was determined to be 2.184. Substituting the value of  $G$  in eqn (S4) gives eqn (S5),

$$FA = -1.767 + \frac{3.505}{2.267 - FA'} \quad (S5)$$

According to the mathematical evaluation of the standard deviation from the propagation of uncertainty, the standard deviation of FA,  $\sigma$ , could be expressed by eqn (S6)

$$\sigma = \sqrt{\left(\frac{\partial(FA)}{\partial(FA')}\sigma'\right)^2} \quad (S6)$$

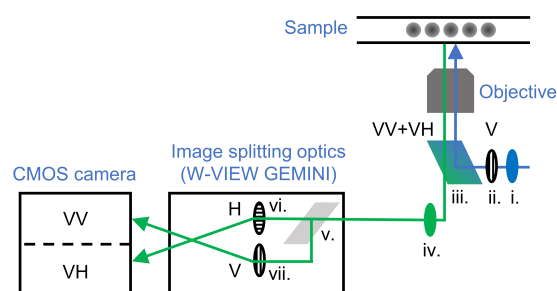
Therefore, the theoretical encoding number  $n$  could be estimated according to eqn (S7),

$$n = \frac{\Delta FA}{4\sigma} \quad (S7)$$

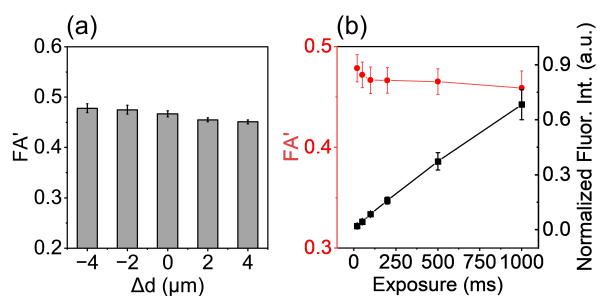
where  $\Delta FA$  is the dynamic range of FA labels and  $\sigma$  is the related average standard deviation. With a dynamic range ( $\Delta FA$ ) of 0.6 and in the current experimental conditions ( $G = 2.184$ ,  $\sigma = 6.44 \times 10^{-3}$ ), the theoretical encoding number was determined to be ca. 23.

**Table S1** The sequences of oligonucleotides used in the DNA assay

Name	Sequence
Target	5'-AAGAGCTGCAAGACCGCGAGG-3'
Probe	5'-C6-SH-CCTCGCGGTCTTGCAGCTCTT-3'

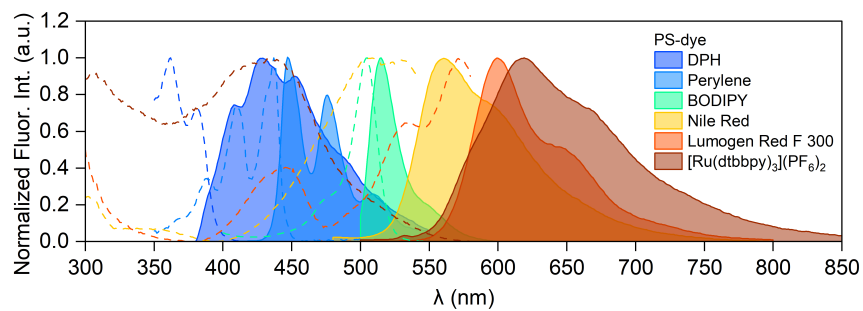


**Fig. S1** Schematic illustration of fluorescence anisotropy imaging microscopy platform (FAIM) used for FA-encoded microspheres (W-VIEW mode, i. excitation filter; ii., vi., and vii. polarizers with relative vertical (V) or horizontal (H) orientations with reference to the optical table surface; iii. dichroic mirror; iv. emission filter; v. 50:50 nonpolarizing plate beamsplitter). In bypass mode, the image is projected directly to the camera without the polarizers and beamsplitter.



**Fig. S2** Influencing factors of FA' in FAIM measurements

(a) Average FA' values of encoded microspheres when adjusting the height of the focal plane (fixing the finest image as the reference distance of 0); (b) Average FA' values and the normalized fluorescence intensity of encoded microspheres when increasing exposure time from 10 to 1000 ms (without overexposure). Sample: PS microspheres doped with 0.04% w/w DPH.



**Fig. S3** Normalized fluorescence spectra of PS microspheres doped with different fluorescent labels (dye/PS w/w = 0.04 %, dashed lines: excitation, color-filled lines: emission).