Supplementary Information for

## Quantitative Evaluation of Analyte Transport on Microfluidic Paper-Based Analytical Devices (µPADs)

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# Calculation of relative amount of applied analyte contributing to colorimetric signal: analyte transport efficiency (on the example of Ni<sup>2+</sup> data shown in Fig. 2a)

According to eq. (1) of the main text, the relative amount of transported  $Ni^{2+}$  [%] is calculated as follows:

$$Transported Ni^{2+}[\%] = \frac{Ni^{2+} in detection area [pmol]}{Ni^{2+} in applied sample [pmol]} \times 100$$
(1)

According to the linear curve fit shown in Fig. 2a, the amount of Ni<sup>2+</sup> reaching the detection area [pmol] is expressed as y = 6.09 x, with x representing the concentration of Ni<sup>2+</sup> in the applied sample liquid (0 – 150  $\mu$ M). On the other hand, the absolute amount of Ni<sup>2+</sup> in the applied sample liquid (20  $\mu$ L) is represented by 20 x [pmol]. Therefore, the Ni<sup>2+</sup> transportation efficiency value is calculated as:

Transported 
$$Ni^{2+}$$
[%] =  $\frac{Ni^{2+} in detection area [pmol]}{Ni^{2+} in applied sample [pmol]} \times 100 = \frac{6.09x}{20x} \times 100 = 30.45\%$ 



Fig. S1 Schematic outline of the experimental procedures used to quantify amounts of transported model analytes: (a) direct colorimetric quantification (applied to  $Ni^{2+}$  and FITC-labelled BSA); (b) indirect absorption spectrometry-based approach (applied to  $Ni^{2+}$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $PO_4^{3-}$ , amaranth and sulforhodamine B).



Fig. S2 Determination of fiber direction based on color spot: (a) actual image of an elliptic color spot; (b) Measurement of spot diameter. Red colored solution (0.02wt% sulforhodamine B aqueous solution; sample volume: 50.0  $\mu$ L) was pipetted onto five different spots of an A4 filter paper. In all cases, the observed color spots were elliptically shaped in a specific direction. Diameters of color spots were measured by Image J. The direction of the longer diameter was regarded as the "machine direction" (MD), while the perpendicular direction (shorter drop diameter) was identified as the "cross direction" (CD).



Fig. S3 Experimental setup for the evaluation of time-dependent liquid travel distances: (a) dimensions of the L-shaped  $\mu$ PAD; the width of the channel parallel to MD was varied by changing the printing preset values (PowerPoint file); channel widths parallel to CD were adjusted to fit those oriented along MD using the calibration data shown in Fig. S4a to cope for cellulose fiber orientation-dependent differences in wax diffusion during heating (see Fig. S5b); (b) observation stand for liquid flow monitoring; (c) snapshot during experimental evaluation of liquid travel distance. Unless otherwise noted, all time-dependent liquid travel distances reported in the main text were measured for channels oriented along MD.



Fig. S4 Relationship between printing preset values (PowerPoint file) and actual dimensions for wax printing-based fabrication of microfluidic structures: (a) channel width; (b) channel length. The actual dimensions were measured using a digital microscope. Channels were fabricated along CD.



Fig. S5 Effect of cellulose fiber orientation: (a) time-dependent liquid travel distance in microfluidic channels aligned with the machine direction (MD) or cross direction (CD); (channel width: 2.0 mm, channel length: open, applied sample volume: 20.0  $\mu$ L); (b) influence of cellulose fiber orientation on the width of microfluidic channels fabricated by the wax printing method, caused by differences in molten wax diffusion speeds.



Fig. S6 Reagents used for the evaluation of Ni<sup>2+</sup> transport: (a) Nitro-PAPS; (b) PAH.

	Before sample application	After sample application
Without PAH		
With PAH		

Fig. S7 Electrostatic immobilization of Nitro-PAPS on  $\mu$ PAD detection areas. Even after sample application, Nitro-PAPS was not washed out by sample flow. Cationic PAH worked as effective immobilizer for anionic Nitro-PAPS on paper.



Fig. S8 Color change of Nitro-PAPS on paper spot test used as calibration data for Ni<sup>2+</sup> transport quantification: (a) actual images of spots after sample application; figures below each spot indicate the absolute amount of Ni<sup>2+</sup> deposited; (b) corresponding calibration curve obtained by digital color analysis; test area: 7.0 mm × 7.0 mm; sample: 0-200  $\mu$ M NiCl<sub>2</sub>; applied sample volume: 5.0  $\mu$ L.



Fig. S9 Comparison between direct colorimetric quantification (RGB-spot test according to Fig. S1a) and absorption spectrometry-based approach (ABS-spot test according to Fig. S1b) for the estimation of transported amounts of Ni<sup>2+</sup>.



Fig. S10 Color of BSA-FITC on paper spot test used as calibration data for BSA transport quantification: (a) actual images of spots after sample application; figures below each spot indicate the absolute amount of BSA deposited; (b) corresponding calibration curve obtained by digital color analysis; test area: 7.0 mm × 7.0 mm; sample: 0-200  $\mu$ M BSA-FITC; applied sample volume: 5.0  $\mu$ L.



Fig. S11 Actual images of entire  $\mu$ PADs after application of 20.0  $\mu$ L samples with varying Ni<sup>2+</sup> (a) or BSA-FITC (b) concentrations; data identical to the one shown in Fig. 2 of the main text.

(a)



Fig. S12 Influence of microfluidic channel width and length on analyte transport: (a) scatter plot version of Fig. 3a, showing channel dimension-dependent transport of Ni<sup>2+</sup>; (b) scatter plot version of Fig. 3b, showing channel dimension-dependent transport of BSA-FITC; (c) channel area-dependent transport of Ni<sup>2+</sup>; (d) channel area-dependent transport of BSA-FITC.



Fig. S13 Actual images of entire  $\mu$ PADs after application of 20  $\mu$ L 100  $\mu$ M Ni<sup>2+</sup> (a) or 50  $\mu$ M BSA-FITC (b) samples; data identical to the one shown in Fig. 3 of the main text.



Fig. S14 Effect of sample liquid viscosity on flow velocity: (a) time-dependent travel distance of aqueous liquids of varying viscosity; (b) fitting to the Washburn equation showing the linear relationship between liquid travel distance (at 90 s as an example) and  $1/\sqrt{\mu}$  ( $\mu$ : viscosity); for viscosity values of glycerol/water mixtures, refer to Table S1; channel width: 2.0 mm, channel length: open, applied sample volume: 20.0 µL.

Table S1 Viscosity of glycerol/water mixtures at 25°C.<sup>1</sup>

Glycerol [wt%]	0	5.0	10.0	15.0	20.0
Viscosity [cP]	0.87	0.99	1.12	1.29	1.49



Fig. S15 Time-dependent travel distance of aqueous solutions in the absence or presence of BSA; the solid red line has been calculated from the Washburn equation based on the BSA-free experimental data and the following values for viscosity and surface tension: water: 0.87 cP,<sup>1</sup> 72.01 mN/m;<sup>2</sup> 50  $\mu$ M BSA: 1.00 cP,<sup>3,4</sup> 57.5 mN/m;<sup>5</sup> channel width: 2.0 mm, channel length: open, applied sample volume: 20.0  $\mu$ L.





Fig. S16 Sample volume capacity of microfluidic channel: (a) 2-area flow channel without wicking area; (b) 3-area flow channel with wicking area. The wicking area contributes to increased capacity of sample flow to the sensing area. In part (b), 25.0  $\mu$ L of sample (largest tested volume) is continuously transported to the signal detection area without cease of capillary action in the presence of the wicking area.



Fig. S17 Time-dependent travel distance of aqueous solutions at different applied sample volumes (10.0, 15.0, 20.0  $\mu$ L). Wicking behavior is not significantly changed by the applied sample volume, as long as the sample is transported by capillarity without exhaustion. Hydrostatic pressure exerted by a stationary sample droplet in the  $\mu$ PAD inlet area is ignorable at least in the range of 10.0-20.0  $\mu$ L of sample volume; channel width: 2.0 mm; channel length: open.



Fig. S18 Actual images of entire  $\mu$ PADs after various sample application methods; data identical to the one shown in Figs. 4a, b, c (for S18a, b, c) and Figs. 5a, b (for S18d, e) of the main text, respectively.

Analuta	Volume of	Sample composition in microplate well		
Analyte	solvent	Eluent	Indicator	
Ni <sup>2+</sup> (counter anion: Cl <sup>−</sup> )	200 μL	180 μL	20 µL	
$Ni^{2+}$ (counter anion: $NO_{3}^{-}$ )	200 μL	180 μL	20 μL	
Zn <sup>2+</sup>	200 μL	180 μL	20 µL	
Cu <sup>2+</sup>	200 μL	180 μL	20 µL	
PO <sub>4</sub> <sup>3-</sup>	200 μL	150 μL	50 μL	
Amaranth	250 μL	200 μL	_	
Sulforhodamine B	250 μL	200 μL	_	

Table S2 Detailed conditions for indirect absorption spectrometry-based method.

After cutting out the paper spot with the dried sample, NiCl<sub>2</sub>, Ni(NO<sub>3</sub>)<sub>2</sub>, ZnCl<sub>2</sub>, CuCl<sub>2</sub> and NaH<sub>2</sub>PO<sub>4</sub> were rehydrated in MES buffer (100 mM, pH 6.5), whereas amaranth and sulforhodamine B were rehydrated in ultrapure water. The elution solvent was chosen to be the same as the sample solvent except for  $PO_4^{3^-}$ . In the case of  $PO_4^{3^-}$  determination, ultrapure water was used as the elution solvent instead of MES buffer (pH 6.5), because an acidic condition is required for the colorimetric reaction.

#### Preparation of colorimetric indicator for ABS-spot tests

The colorimetric indicator solution used for Ni<sup>2+</sup>, Zn<sup>2+</sup> and Cu<sup>2+</sup> is a 500  $\mu$ M Nitro-PAPS solution prepared in 100 mM MES buffer at pH 6.5. Determination of PO<sub>4</sub><sup>3-</sup> is based on the color change of ammonium molybdate and malachite green under acidic condition.<sup>6</sup> The colorimetric indicator solution for PO<sub>4</sub><sup>3-</sup> was prepared by mixing three reagents A, B and C (A: 1.0 mM malachite green oxalate and 1.0 wt% PVA aqueous solution; B: 350 mM hexaammonium heptamolybdate tetrahydrate aqueous solution; C: commercially available concentrated 98.0% sulfuric acid). After mixing 10.0 mL of reagent A, 10.0 mL of reagent B and 5.0 mL of reagent C followed by 30 min stirring, the supernatant liquid was used as the colorimetric indicator for PO<sub>4</sub><sup>3-</sup>. This indicator changes from yellowish green to dark green with increasing concentration of PO<sub>4</sub><sup>3-</sup>. Note that no colorimetric indicator is required for amaranth and sulforhodamine B since their inherent absorbance (maxima at 522 and 565 nm, respectively) was utilized as the optical detection signal.



0.45 Absorbance at 568 nm [-] y = 0.000256 x + 0.102 R<sup>2</sup> = 1.00 0.35 0.25 0.15 *n* = 5 0.05

250

0

500

Ni(NO<sub>3</sub>)<sub>2</sub> [pmol]

750

1000

φ



(a)

(d)





CuCl<sub>2</sub> [pmol]





Fig. S19 Calibration data obtained by the indirect absorption spectrometry-based approach (see Fig. S1b): (a) NiCl<sub>2</sub>; (b) Ni(NO<sub>3</sub>)<sub>2</sub>; (c) ZnCl<sub>2</sub>; (d) CuCl<sub>2</sub>; (e) NaH<sub>2</sub>PO<sub>4</sub>; (f) amaranth; (g) sulforhodamine B; applied sample volume: 5.0  $\mu$ L.



(b)

(a)



Fig. S20 Chemical structures of (a) amaranth and (b) sulforhodamine B.



Fig. S21 Visualization of metal ion (Ni<sup>2+</sup>, Zn<sup>2+</sup>, and Cu<sup>2+</sup>) transport on  $\mu$ PADs with a straight detection paper channel; inlet area: 7.0 mm × 7.0 mm; channel width: 2.0 mm; channel length: 18.0 mm. Nitro-PAPS and PAH were deposited on the flow channel under identical condition to the standard  $\mu$ PADs used throughout this work. After deposition of 20.0  $\mu$ L of Ni<sup>2+</sup>, Zn<sup>2+</sup> or Cu<sup>2+</sup> sample solution (100  $\mu$ M) onto the inlet area, different lengths of the color-changed area (Ni<sup>2+</sup> > Zn<sup>2+</sup> > Cu<sup>2+</sup>) were observed depending on the affinity of the metals to cellulose (Ni<sup>2+</sup> < Zn<sup>2+</sup> < Cu<sup>2+</sup>).

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