Supporting Information for:

Disposable paper strips for carboxylate discrimination

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Instrumental parameters

Measurements on solid supports were conducted on a Biotek Synergy II multimode microwell plate reader, using standard protocols developed for plastic 96-well plates. The instrument uses a tungsten lamp for excitation, and bandpass filters for wavelength selection. In the top-detected fluorescence emission mode used here, excitation light was directed to the substrate spot from the top side, and emitted light was collected from the same side, thanks to a dichroic mirror placed between the sample and the emission channel. Measurements were conducted at room temperature.

- Excitation filters used: 450 ± 25 nm, 460 ± 20 nm, 485 ± 10 nm
- Emission filters: 516 ± 25 nm, 528 ± 25 nm, 560 ± 25 nm, 580 ± 25 nm
- Dichroic mirror: 510 nm cut off (i.e. reflective for $\lambda < 500$ nm; transparent for $\lambda > 520$ nm)

Even on our unusual paper-supported samples, we were still able to use the instrument's automated gain adjustment routines for its emission detector so that the strongest fluorescence emission on each plate reached 85% of the instrument's full scale. The automatically determined best values of the gain setting used for each experiment / support are reported below.

- Detector gain for calcein binding & displacement (λ_{exc} = 485 nm; λ_{em} = 516 nm): 38
- Detector gain for "shelf life" experiment (λ_{exc} = 485 nm; λ_{em} = 516 nm): 38

Table S1. Fluorescence detector gain for differentiation experiments

Gain	Fig. 4a:	Fig. 4b & S3:	Fig. 5:	Fig. 6:	Fig. 7 & 8	Fig. S2:	Fig. S4:
450/516	41	33	39	39	40	36	38
450/528	40	32	39	38	40	36	37
450/560	40	33	40	39	41	37	38
450/580	44	36	45	42	45	41	41
460/516	38	31	38	37	38	34	36
460/528	37	30	38	37	38	34	36
460/560	40	32	39	37	39	35	36
460/580	42	35	43	41	43	39	40
485/516	35	31	35	35	36	33	34
485/528	35	30	35	35	35	34	34
485/560	36	31	37	36	37	35	35
485/580	40	34	41	39	40	38	38

Fig. 4a: office printer paper (first attempt, direct one-step deposition)
Fig. 4b: office printer paper, two-step deposition (further exp. use two-step deposition as well)
Fig. 5: filter paper support (citrate, isocitrate, malate, oxaloacetate, and tricarballylate)
Fig. 6: chromatography paper (citrate, isocitrate, malate, oxaloacetate, and tricarballylate)
Fig. 7&8: chromatography paper (above anions + hydroxyanions: lactate, tartrate, glycolate)
Fig. S2: TLC plates
Fig. S3: printer paper, two-step deposition (wet or dry sample measurements considered separately)
Fig. S4: chromatography paper (citrate, isocitrate, malate, and oxaloacetate)

Dye screening

Several dyes (shown in Figure S1) that were good PAMAM-binding candidates were taken into consideration. They were studied and eliminated due to different reasons. For example, binding between alizarin red S and PAMAM caused only a very small change in the spectroscopic signal of the dye, providing very limited dynamic range, which would not be ideal for displacement. Glycine cresol red bound well to PAMAM but provided very poor differentiation. Therefore, those dyes listed below were no longer considered.

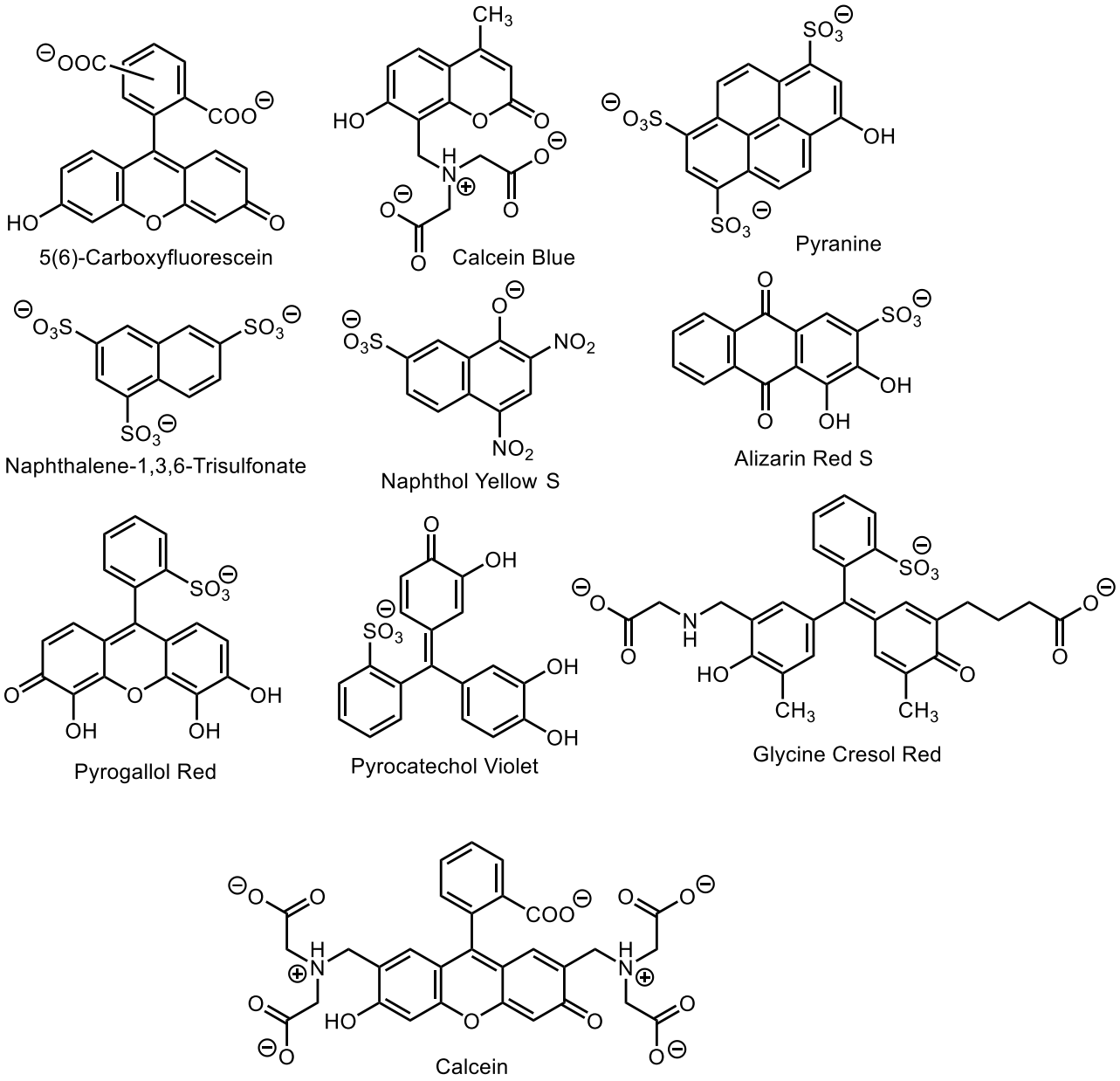

Figure S1. Anionic organic dyes considered for screening

Binding of calcein to PAMAM G5 in solution

Binding between the calcein dye and ethylenediamine-core amine-terminated fifth-generation poly(amidoamine) dendrimer (G5 PAMAM) was first ascertained in solution. Measurements were conducted on an ISS PC50 steady-state spectrofluorometer. A broad-spectrum high-pressure xenon lamp (CERMAX, 300W) was used for excitation; the excitation was corrected by diverting a portion of the excitation beam through a rhodamine B quantum counter and a dedicated detector. The excitation intensity was controlled by a manually operated iris (set to open here) and slits (slits for excitation and emission were set for 2 nm spectral resolution). The emitted light was detected by a Hamamatsu red-sensitive PMT, operating in photon counting mode. The temperature of the cuvette was fixed to 25°C by an external circulating water bath.

To a quartz cuvette containing 2 mL of 6.36 μM calcein solution in HEPES buffer were added small aliquots of a concentrated G5 amine-terminated PAMAM solution in the same medium until no further change was observed in the emission behaviour of the resulting solution. Upon excitation at 494 nm, the emission intensity at 518 nm was measured as a function of the added dendrimer concentration. The fluorescence intensity typical of calcein at 518 nm first decreased sharply, followed by an increase to a plateau upon further additions of PAMAM, as shown in Figure S2 below.

The sharp decrease in fluorescence emission towards the beginning of this titration is due to self-quenching of the dye; in fact, multiple molecules of dye bind to the same molecules of dendrimer when the total dendrimer concentration is low and the dye in present in relative excess with respect to it. Due to the small Stokes shift typical of xanthene-type dyes such as cyanine, in these conditions the dendrimer-bound dye molecules end up close enough to each other to trigger efficient resonance energy transfer (RET), leading to extensive quenching of their emission. This is typical of the binding profiles of xanthene-type dyes binding to these dendrimers and was observed in our previous studies of similar dyes as well.[1] This profile unambiguously indicated to us that calcein binds to the PAMAM dendrimer in aqueous environment.

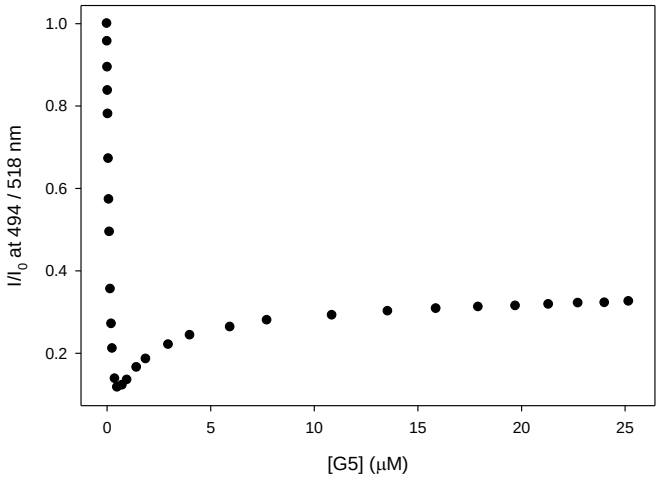

Figure S2. Binding isotherm obtained from the titration of calcein with PAMAM G5 in solution. [calcein] = 6.36 μM in H_2O buffered with 50 mM HEPES buffer at pH 7.4.

Limit of detection

Figure S3 below shows the determination of the limit of detection (LOD) for the [calcein•PAMAM] complex as a sensor for citrate on chromatography paper, as a sample carboxylate anion. On a chromatography paper paper with 96 spots, a solution of [calcein•PAMAM] complex sensor that contained 63.6 pmol of calcein and 21.3 pmol of PAMAM dendrimer was first deposited and allowed to dry. Then, increasing amounts of citrate were deposited from solution, in 8-replicate. The concentration range was determined from Figure 3 (see manuscript), which shows that the citrate binding profile remained linear over the at 0 to 2 nanomoles range of citrate amounts. The plate was then read by a plate reader as described above.

From the profile shown below, the signal for citrate detection is linear over this concentration range and can be fit to the linear expression $y = 5182\ x + 23348$, with a $r^2 = 0.971$. To determine the LOD we used the customary threshold level of background value (= bound dye in this case) + 3x the standard deviation of the blank, i.e. 23348 + 3 * 973 = 26267 in this case; the corresponding citrate amount is 0.56 nanomoles.

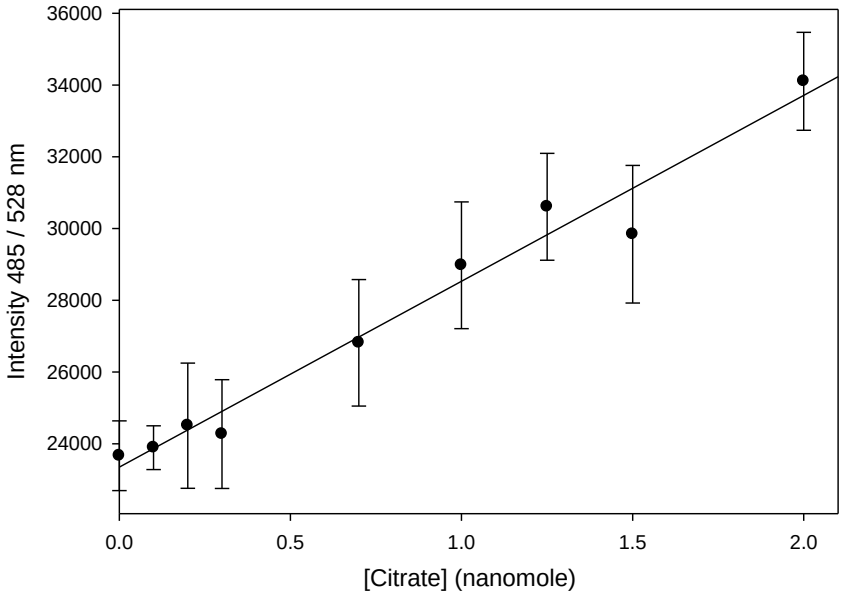

Figure S3. Limit of detection (LOD) for the [calcein•PAMAM] complex as a sensor for citrate on chromatography paper. The value of each point is the calculated average of 8 replicates; error bars for each value are included as well. Excitation: 485 nm, emission: 528 nm, calcein = 63.6 pmol, PAMAM = 21.3 pmol in each spot.

Carboxylate differentiation on TLC plates

Differentiation of citrate, isocitrate, oxaloacetate, and maleate using the [calcein•PAMAM] complex as a sensor is shown below. 190.8 pmol of calcein, 63.9 pmol of PAMAM G5, and 69 nmol of analyte were deposited on each spot. The plate was read after the solutions had dried; data collection and processing methods were the same as described in the main manuscript for other supports. The results are shown in Figure S4 below as an LDA scores plot. Unfortunately, the dispersion of the replicates in each cluster was very large, and of a magnitude comparable to the distance between the clusters, resulting in overlapping clusters and poor differentiation. For this reason, this support was not used further.

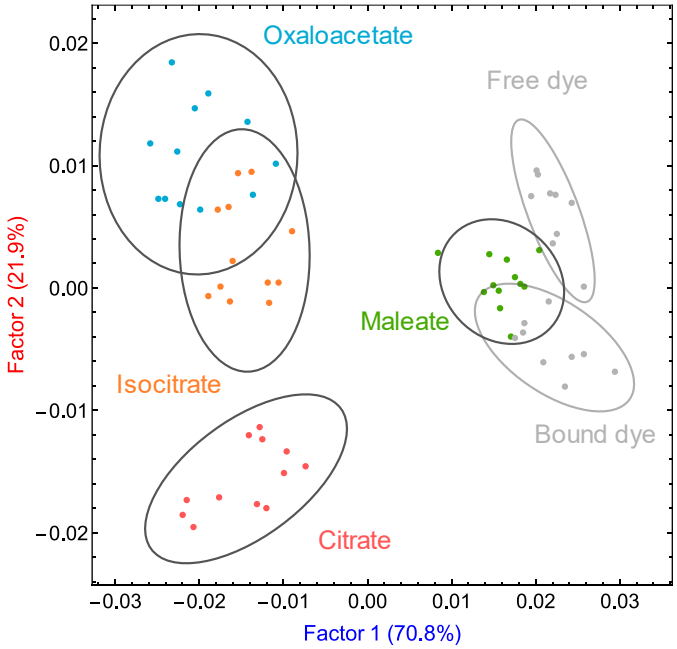

Figure S4. LDA scores plots obtained for the differentiation of citrate, isocitrate, maleate, and oxaloacetate by the [calcein•PAMAM] complex as sensor on TLC plate as solid support.

Carboxylate differentiation on printer paper: "wet" *or* "dry" spots only

Results from the differentiation of citrate, isocitrate, oxaloacetate, and maleate using the [calcein•PAMAM] complex as a sensor on a printer paper plate are shown below. 636 pmol of calcein, 213 pmol of PAMAM G5 were deposited on a printer paper plate and allowed to dry, then 230 nmol of each analyte were deposited on the corresponding spots. The plate was first read immediately after analyte deposition (result shown in Figure S5a, "wet" samples). The same plate was then left to stand in air for 2 hours, after which time the samples had dried completely; the measurement was then repeated, with the result shown in Figure S5b ("dry" samples). The data collection and processing methods described in the main manuscript for this support were used in this case as well.

The "wet plate" discrimination results were regrettably poor (Figure S5a), with little usable differentiation. The "dry plate" data showed better separation on its own, although significant overlapping among some anion clusters was still observed. Surprisingly, when data from both measurements was combined, printer paper supports were able to discriminate the four carboxylates; this result is reported in Figure 4b in the main manuscript.

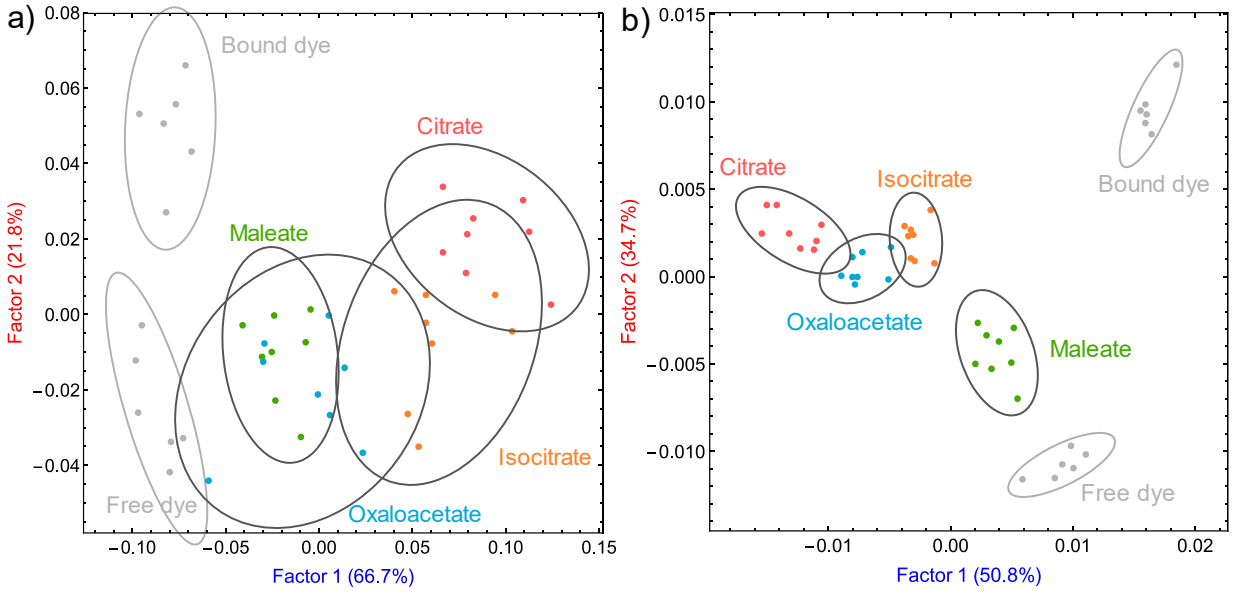

Figure S5. LDA scores plots obtained for the differentiation of citrate, isocitrate, maleate, and oxaloacetate by the [calcein•PAMAM] complex as sensor on printer paper as solid support. Left: plate was measured immediately after sample deposition (samples remained wet); right: results from *the same plate* after it was allowed to dry for 2 hours, at which point the samples had dried completely.

Comparing chromatography paper to filter paper supports

To directly compare the performance of chromatography paper to that of printer paper, an experiment was run on chromatography paper using the same set of carboxylates that had been previously analysed on printer paper. The differentiation of citrate, isocitrate, oxaloacetate, and maleate using the [calcein•PAMAM] complex as a sensor was thus attempted on a chromatography paper plate: 159 pmol of calcein, 53.25 pmol of PAMAM G5, and 57.5 nmol of carboxylate analyte were deposited on each sensor spot on a chromatography paper plate from solution, using the two-step deposition method (i.e. calcein and PAMAM was deposited first; this was allowed to dry, then carboxylates were deposited on the same spots). The plate was read after the samples had dried (10 min.); data collection and processing described for other supports was used here as well.

Chromatography paper (Figure S6) shows clear advantages compared to printer paper (Figure 4b of the main manuscript). With better separation of analytes, larger intercluster distances (i.e. clusters are further apart), and smaller intracluster distances (i.e. tighter clusters); chromatography paper supports allows the [calcein•PAMAM] sensing system to captures more information, generating a more nuanced differentiation among analytes, whereas the LDA scores plot for printer paper plates (Figure 4b) had all analytes squeezed to the left side of the plot. Indeed, in the latter case factor 1 did not differentiate among the analytes, rather only separating all analytes from the reference clusters.

From a practical standpoint, chromatography paper also required 3x less material than printer paper; and samples on chromatography paper dried within 10 minutes, while those deposited on printer paper required 2 hours (!). Finally, the results obtained on chromatography paper in "dry sample" conditions, i.e. after analyte solutions have fully evaporated, are enough for differentiation, while "wet" and "dry" measurements from printer paper supports had to be combined to obtain viable differentiation.

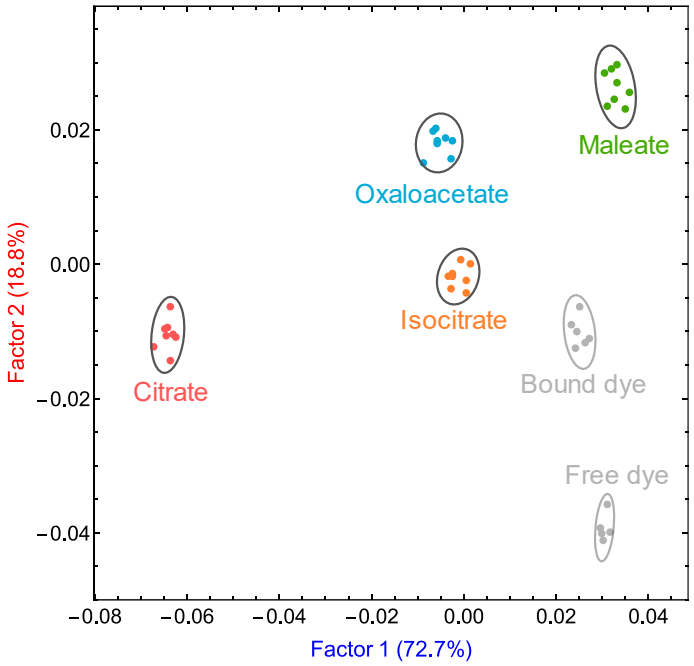

Figure S6. LDA scores plots obtained for the differentiation of citrate, isocitrate, maleate, and oxaloacetate by the [calcein•PAMAM] complex as sensor on chromatography paper as solid support.

Outlier determination and elimination for pattern recognition study

In all measurements on solid support, multiple steps were taken to condition the data for LDA analysis. First, data was acquired from the plate reader; then, data corresponding to poor combinations of excitation / emission filters for the experiment at hand were removed by inspection, to prevent the inadvertent injection of noise in the system.

Outlier tests were then performed among the groups of replicates for each analyte before further analysis. A representative example of such analysis is shown in Figure S7 below using the glycolate replicates from the discrimination of hydroxy carboxylates on chromatography plate (shown in Figure 7 of the main manuscript). Data for the 36 replicates of the glycolate analyte was subjected to Principal Component Analysis (PCA);[2] the scores along the first two components obtained from analysis were retained and the replicates plotted in a 2D PCA scores plot, together with the 95% multivariate confidence ellipsoid calculated from the coordinates of these points (dotted ellipse in Figure S3 below). The replicate corresponding to the dot shown in red (replicate #17) was found very far from the rest of the distribution and removed. In our experience, typical causes for such errant points are imperfections in the sample support (e.g. due to warping or crosstalk); in some cases, they were caused by inadvertent mistakes in the manual sample deposition, particularly when depositing very small liquid volumes using multichannel pipettes.

The same analysis was repeated for the group of replicates obtained for each analyte. After outlier rejection, the resulting data set was subjected to LDA analysis.

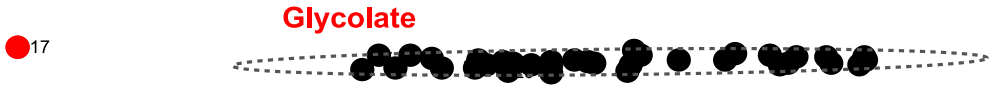

Figure S7. A representative example of outlier rejection from the replicates obtained for glycolate anion on chromatography paper support. The dotted ellipse shows the multivariate confidence interval at 95% confidence; black dots are replicates that falls within this interval, while red dots indicate outlier candidates for possible removal.

LDA loadings plots for carboxylate differentiation on chromatography paper

A loadings plot summarizes the contribution of each raw instrumental variable to the differentiation along each factor.[3-4] Below is an example of such a plot, from the discrimination of hydroxy carboxylates on chromatography paper plate, whose corresponding LDA scores are shown in Figure 8a in the main manuscript. Each point in this plot corresponds to a raw measurement channel, identified by the respective excitation and emission wavelengths. The coordinates of each point on the loadings plot describe the contribution of that instrumental measurement to each factor, the abscissa being the contribution to factor 1, and the ordinate the contribution to factor 2, respectively.

For example, the point labelled "485/528 (21, 0)" should be interpreted as follows: information contained in the fluorescence intensity measurements obtained at 528 nm with excitation at 485 nm contributed 21% of the total information explained by that factor; on the other hand, this variable contributed no information to factor 2, as indicated by its 0 ordinate value. Similarly, intensity measurements at 560 nm upon excitation at 450 nm contributed 31% of the information carried by factor 2, and none to factor 1. Some measurements contribute to both factors to varying extents.

The fact that multiple variables contribute to each one of the two factors shows that the analytical discriminatory power of these systems is inherently multivariate, i.e. it does not rely on any one measurement, but on information from multiple ones for an effective differentiation.

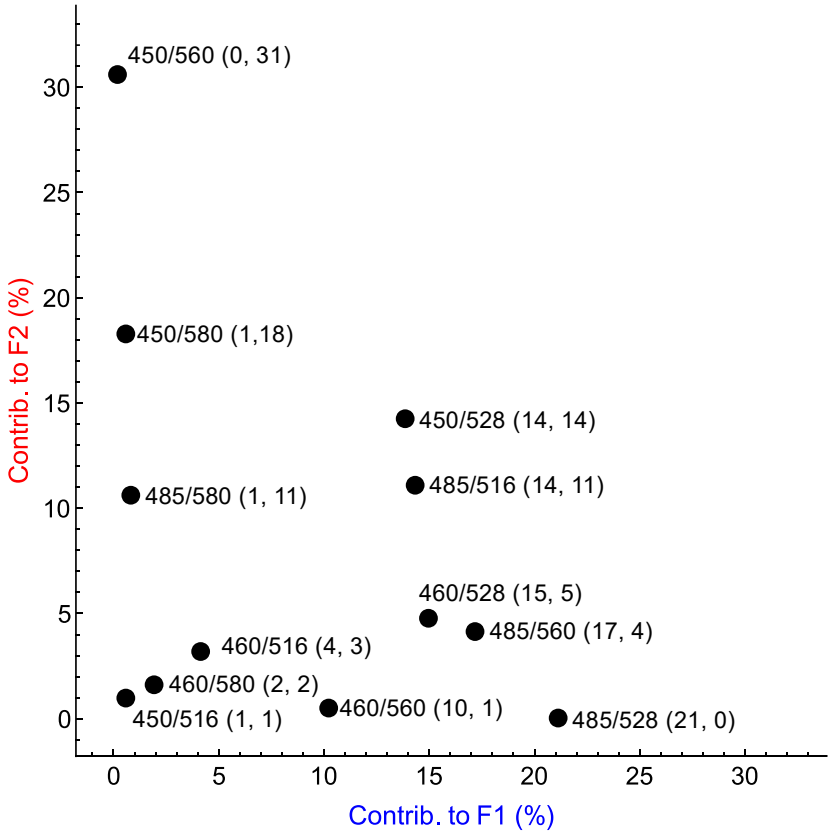

Figure S8. Loadings plot for LDA analysis from the discrimination of hydroxy-containing carboxylate anions on chromatography paper plates; each variable is labelled with the corresponding $\lambda_{exc}/\lambda_{em}$.

Cost analysis

We carried out a Cost analysis for the screening of 96 samples on a 96-well polystyrene plate with black walls (designed for fluorescence, and commonly used in anion sensing and recognition), versus running the same samples on printer paper, filter paper, and chromatography paper, respectively. The results are summarized in Table S2 below. Running the assay on a plastic microwell plate has an estimated cost of $3.56, whereas the paper supported ones cost significantly less, at $0.01, $0.25, and $0.32, respectively.

Differences in the price are primarily due to the cost of the support. Secondly, the 96-well plate assay uses 300 μL of solution containing the costly dendrimer for each well; on the other hand, the solid supports only need 1-10 μL of the same solution per spot, depending on the specific support material used. Even though the spotting solution prepared for solid support deposition was 10-fold more concentrated than the one used in the solution assay on the microwell plate, to speed up drying, nevertheless the overall amount of each component needed was still found to be much less on solid support than in microwell plates.

Table S2. Cost analysis

material	support ($)	calcein (g)	PAMAM G5 (g)	HEPES (g)	total cost ($)
Unit cost ($)		$7.325/g	$346/g	$0.313/g	
96-well plate (black wall)	$ 3.573	122 μg	41 μg	960 μg	$ 3.588
Printer paper plate	$ 0.004	41 μg	14 μg	32 μg	$ 0.009
Filter paper plate	$ 0.247	4 μg	1.4 μg	3.2 μg	$ 0.248
Chromatography paper plate	$ 0.323	10 μg	3.4 μg	8 μg	$ 0.324

References

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