Fluorescence crosstalk reduction by *modulated excitation-synchronous acquisition* for multispectral analysis in high-throughput droplet microfluidics.

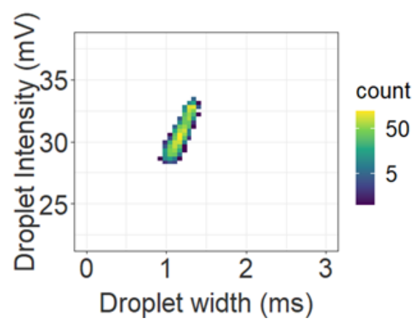
Jatin Panwar [a, b] & Christoph A. Merten [a, #]

[a] Institute of Bioengineering, School of Engineering, École Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland

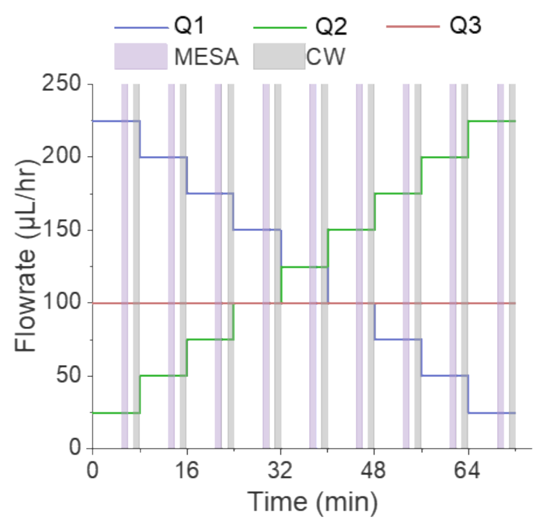
[b] European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

Correspondence: christoph.merten@epfl.ch

Supplementary Information :



Supplementary Figure S1: *Distribution of droplet width (time taken by the droplet to cross the detection point) and the corresponding fluorescence intensity of 0.3 µM CB acquired in channel 2 (from the experiment shown figure 3a). The mean droplet width is 1.25 ± 0.2 ms.*



Supplementary Figure S2. *Schematic of the LabVIEW algorithm to systematically acquire the droplet fluorescence data at different concentrations of fluorescent dyes in MESA and CW mode. The algorithm controls thee pumps (containing 1 µM cascade blue (CB), 1 µM Fluorescein (FL) and 1x PBS) that are connected to the three aqueous inlets of the microfluidic device shown in* ***Figure 2a,*** *while the fourth inlet for oil is kept at a constant flow rate of 800 µL/hr. The program stepwise increases the fluorophore concentration by controlling the flow rate (Q1, Q2 and Q3) ratio of three aqueous inlets, while keeping the total flow rate as 350 µL/hr. After generating any specific flow rate ratio to reach desired concentrations of the fluorophores in the droplets, the program waits for 5 minutes for flow stabilization. Then the program acquires droplet fluorescence signals in MESA mode for 1 minute after which the mode changes to CW mode and the data is acquired again for 1 minutes after a wait of 1 minute. After the data acquisition in CW mode, the program changes the flow rates to reach the next concentration and the whole operation repeats.*

Cascade Blue Conc. (µM)	f_1^{MESA}	f_2^{MESA}	Std f_1^{MESA}	Std f_2^{MESA}	f_1^{CW}	f_2^{CW}	Std f_1^{CW}	Std f_2^{CW}	$\frac{f_2^{MESA}}{f_1^{MESA}}$	$C_{1\to2}^{MESA}$	Std $C_{1\to2}^{MESA}$	$\frac{f_2^{CW}}{f_1^{CW}}$	$C_{1\to2}^{CW}$	Std $C_{1\to2}^{CW}$	R_{12}
0.1	50.702	0.298	12.087	3.753	31.702	4.090	17.320	4.459	0.006			0.081			
0.2	103.633	1.136	14.748	5.092	80.114	19.698	23.127	6.309	0.011			0.190			
0.3	168.717	0.963	9.598	4.903	167.833	30.455	30.347	7.087	0.006			0.181			
0.4	244.399	2.000	8.875	4.955	234.864	42.927	10.263	3.429	0.008			0.176			
0.5	299.560	1.483	8.853	5.002	290.385	52.871	9.313	4.004	0.005	0.006	0.002	0.176	0.170	0.034	**96.398**
0.6	341.409	1.547	10.123	5.629	359.749	57.196	12.293	3.638	0.005			0.168			
0.7	384.551	2.136	10.981	5.406	379.105	68.432	10.561	4.070	0.006			0.178			
0.8	423.863	1.413	9.899	5.505	432.065	80.124	9.076	3.329	0.003			0.189			
0.9	462.623	2.759	9.627	5.603	461.142	88.043	12.688	4.070	0.006			0.190			

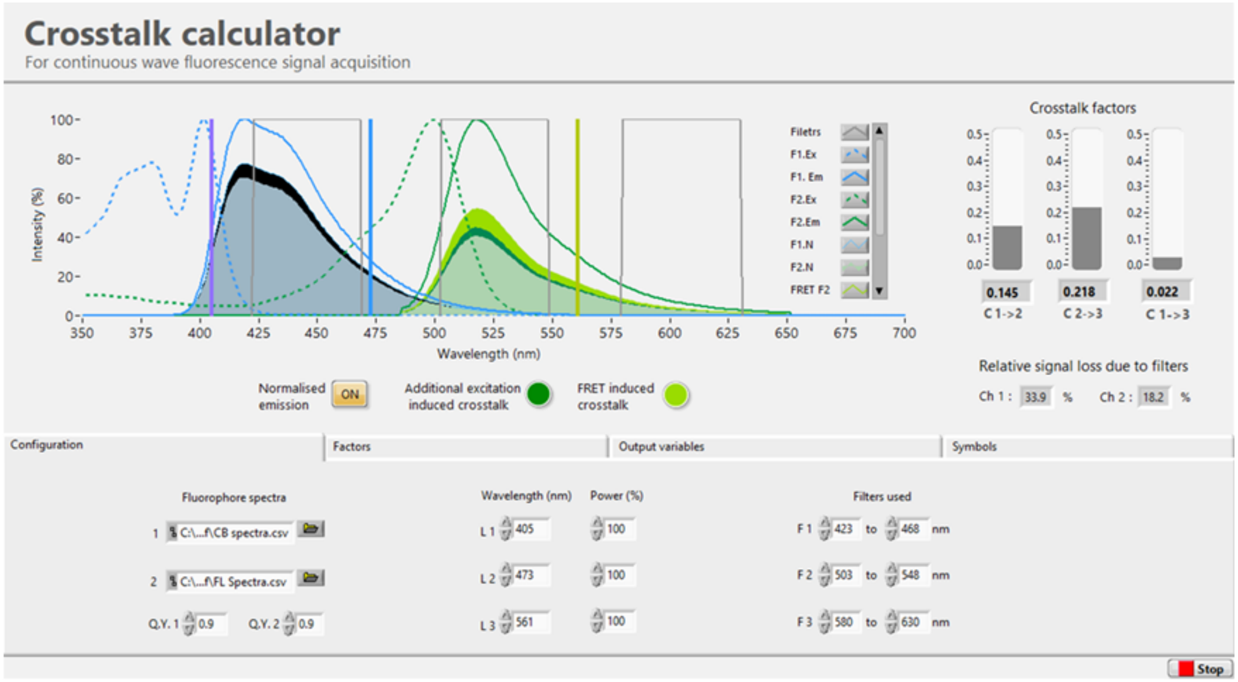
Supplementary Table S1. *Calculation of the crosstalk factor $C_{1\to2}$ using equation 1 and experimentally determined values of droplet fluorescence amplitude (f) at different concentrations of cascade blue with MESA and CW mode. The overall crosstalk reduction (R_{12}), calculated using equation 2 between Ch1 and Ch2 by MESA as compared to CW, is also shown.*

Fluorescein Conc. (µM)	f_2^{MESA}	f_3^{MESA}	Std f_2^{MESA}	Std f_3^{MESA}	f_2^{CW}	f_3^{CW}	Std f_2^{CW}	Std f_2^{CW}	$\frac{f_3^{MESA}}{f_2^{MESA}}$	$C_{2\to3}^{MESA}$	Std $C_{2\to3}^{MESA}$	$\frac{f_3^{CW}}{f_2^{CW}}$	$C_{2\to3}^{CW}$	Std $C_{2\to3}^{CW}$	R_{23}
0.1	38.50	0.26	7.25	0.86	22.93	6.56	10.29	5.13	0.007			0.170			
0.2	75.60	0.09	7.96	1.20	77.38	20.68	6.98	3.97	0.001			0.274			
0.3	118.95	0.58	7.11	1.50	116.07	31.06	2.98	3.35	0.005			0.261			
0.4	171.95	0.69	15.31	1.78	164.01	42.64	2.63	3.36	0.004			0.248			
0.5	205.18	0.90	6.40	1.80	201.42	52.80	7.31	4.36	0.004	0.004	0.0015	0.257	0.251	0.029	**98.315**
0.6	241.67	1.11	8.08	2.37	239.39	63.85	5.40	4.01	0.005			0.264			
0.7	279.68	1.31	7.67	2.44	287.07	74.68	5.37	4.26	0.005			0.267			
0.8	324.84	1.57	12.51	2.82	319.60	82.26	6.80	4.99	0.005			0.253			
0.9	344.51	1.55	12.19	0.56	348.72	88.49	8.76	5.11	0.004			0.257			

Supplementary Table S2. *Calculation of the crosstalk factor ($C_{2\to3}$) and using equations 1 and experimentally determined values of droplet fluorescence amplitude (f) at different concentrations of fluorescein with MESA and CW mode. The overall reduction (R_{23}), calculated using equation 2 between Ch2 and Ch3 by MESA as compared to CW, is also shown.*

Cascade Blue Conc. (µM)	f_1^{MESA}	f_3^{MESA}	Std f_1^{MESA}	Std f_3^{MESA}	f_1^{CW}	f_3^{CW}	Std f_1^{CW}	Std f_3^{CW}	$\frac{f_3^{MESA}}{f_1^{MESA}}$	$C_{1\to3}^{MESA}$	Std $C_{1\to3}^{MESA}$	$\frac{f_3^{CW}}{f_1^{CW}}$	$C_{1\to3}^{CW}$	Std $C_{1\to3}^{CW}$	R_{13}
0.1	44.91	0.02	13.30	0.55	56.75	4.81	5.73	2.57	0.00041			0.107			
0.2	118.37	0.12	17.93	0.51	162.57	7.59	21.52	2.57	0.00100			0.064			
0.3	215.85	0.22	9.08	0.54	237.33	8.10	9.63	1.66	0.00101			0.038			
0.4	298.56	0.27	11.40	0.52	337.88	13.71	18.15	2.61	0.00090			0.046			
0.5	394.04	0.44	16.20	0.56	400.47	17.60	9.34	2.11	0.00111	0.00074	0.000325	0.045	0.052	0.022	**98.568**
0.6	462.20	0.47	10.64	0.57	475.03	20.31	11.20	1.73	0.00101			0.044			
0.7	540.67	0.34	14.27	0.54	535.60	23.09	10.55	1.47	0.00063			0.043			
0.8	601.37	0.16	10.45	0.47	609.41	24.79	8.72	1.15	0.00026			0.041			
0.9	649.64	0.25	6.90	0.48	641.44	26.27	6.32	2.02	0.00038			0.040			

Supplementary Table S3. *Calculation of the crosstalk factor $C_{1\to3}$ using equation 1 and experimentally found values of droplet fluorescence amplitude (f) at different concentrations of cascade blue with MESA and CW mode. The overall crosstalk reduction (R_{13}), calculated using equation 2 between Ch1 and Ch3 by MESA as compared to CW, is also shown.*



$$C_{i \to j} = \frac{\textit{Fluorescence signal in } Ch_{(j)} \textit{ due to } Ch_{(i)}}{\textit{Fluorescence signal in } Ch_{(i)}}$$

Fluorescence signal in Ch(j) due to Ch(i) *= emission from Fluorophore(j) due to Laser(j)* *+ emission from Fluorophore(j) due to Laser(i)* *+ emission gained by acceptor due to FRET* *- emission lost by donor due to FRET*	**Fluorescence signal in Ch2 due to Ch1** $= Em_{12} * Ex_{11} * Lp_1 * Q_1 * Pg_2 * Pr_2 * DL_2 * DL_1' * FL_2$ $+ Em_{22} * Ex_{21} * Lp_1 * Q_2 * Pg_2 * Pr_2 * DL_2 * DL_1' * FL_2$ $+ Em_{22} * Ex_{2FEm1} * Q_2 * Pg_2 * Pr_2 * DL_2 * DL_1' * FL_2$ $- 0$
Fluorescence signal in Ch1 $= Em_{11} * Ex_{11} * Lp_1 * Q_1 * Pg_1 * Pr_1 * DL_2 * DL_1 * FL_1$ $+ 0$ $+ 0$ $- Em_{11} * Ex_{2FEm1} * Q_2 * Pg_1 * Pr_1 * DL_2 * DL_1 * FL_1$	**Fluorescence signal in Ch3 due to Ch2** $= Em_{23} * Ex_{22} * Lp_2 * Q_2 * Pg_3 * Pr_3 * DL_2' * FL_3$ $+ Em_{23} * Ex_{21} * Lp_1 * Q_2 * Pg_3 * Pr_3 * DL_2' * FL_3$ $+ Em_{23} * Ex_{2Fem1} * Q_2 * Pg_3 * Pr_3 * DL_2' * FL_3$ $- 0$
Fluorescence signal in Ch2 $= Em_{22} * Ex_{22} * Lp_2 * Q_2 * Pg_2 * Pr_2 * DL_2 * DL_1 * FL_2$ $+ Em_{22} * Ex_{21} * Lp_1 * Q_2 * Pg_2 * Pr_2 * DL_2 * DL_1 * FL_2$ $+ Em_{22} * Ex_{2Fem1} * Q_2 * Pg_2 * Pr_2 * DL_2 * DL_1 * FL_2$ $- 0$	**Fluorescence signal Ch3 due to Ch1** $= Em_{13} * Ex_{11} * Lp_1 * Q_1 * Pg_3 * Pr_3 * DL_2' * FL_3$ $+ Em_{23} * Ex_{21} * Lp_1 * Q_2 * Pg_3 * Pr_3 * DL_2' * FL_3$ $+ Em_{23} * Ex_{2FEm1} * Q_2 * Pg_3 * Pr_3 * DL_2' * FL_3$ $- 0$

$$Ex_{2FEm1} = Em11 * Ex11 * Lp1 * Q1 * \frac{\textit{Area of overlap between exctation of fluorophore 2 with emission of fluorophore 1}}{\textit{Wavelength span of the overlap}}$$

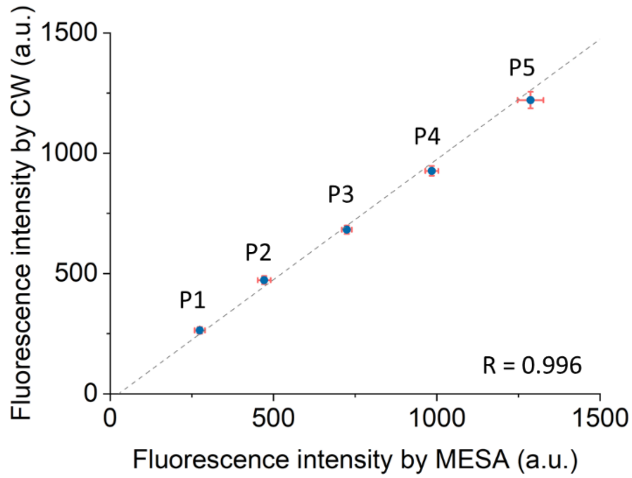
Supplementary Figure S3: *Screenshot of the interactive crosstalk calculator tool developed to simulate and theoretically calculate the crosstalk factors for different configuration of fluorophores, lasers and filters used in conventional continuous wave fluorescence analysis setups. The crosstalk factor ($C_{i \to j}$) is calculated using the equations shown. Here, Em_{ij} = Total emission of F(i) falling in Ch(j), Ex_{ij}= Fraction of F(i) excited by L(j), Lp_i = Power of Laser L(i), Q_i = Quantum yield of fluorophore(i), Pg_i = Gain of PMT(i), Ps_i = Spectral response of PMT(i), DL_i = Fraction of correct signal transmitted/reflected through dichroic mirror(i), DL_i'= 2 – DL_i = Additional fraction of incorrect signal transmitted/reflected through dichroic mirror(i). FL_i = Loss during transmission through filter(i) and Ex_{jFEmi} = Fraction of F(j) excited due to FRET from F(i). In the simulation, these values are either taken from the experiment (like laser power, PMT gain) or from the material datasheet (like fluorophore spectra, quantum yields and PMT's spectral response). To simplify the complex optical setup, we assumed the fraction of signal transmitted through any filter (FL_i) and dichroic mirror (DL_i) as 0.95, which also represents an average value of the manufacturer's data sheet. The rest of the values are calculated by the tool. For e.g. Em_{11} is calculated by integrating the emission spectra of CB from 423 nm to 468 nm that are the wavelengths allowed to transmit through filter 1; and Ex_{11} is calculated by finding the intercept of 405 nm laser on the excitation spectra of CB giving a value of 0.0861 which is multiplied by the CB emission spectra to normalize it.*

	$C_{1\to2}$	$C_{2\to3}$	$C_{1\to3}$
Observed values	0.17 ± 0.0022	0.25 ± 0.029	0.052 ± 0.02
Calculated values	0.145	0.218	0.022

***Supplementary Table S4**. Crosstalk factors calculated theoretically using the interactive tool "crosstalk calculator" (**Supplementary Software1**) in comparison to the crosstalk factors observed experimentally in continuous wave (CW) fluorescence data acquisition.*

Filter configuration	*Filter 1*	*Filter 2*	*Filter 3*	$C_{1\to2}$	$C_{2\to3}$	$C_{1\to3}$	*Relative signal Loss in Ch1 (%)*	*Relative signal Loss in Ch2 (%)*
This paper	445/45	525/45	605/50	0.145	0.218	0.022	33.9	18.2
Reduced spectral overlap	432/45	532/40	610/40	0.094	0.162	0.012	21.2	29.3
Narrow band	432/20	562/20	610/20	0.095	0.149	0.011	60.6	62.9

***Supplementary Table S5.** Theoretically calculated crosstalk factors for various configurations optical filters along with the corresponding fraction of emission acquired by the photomultiplier tubes via the filters. The relative signal loss is calculated as the ratio of usable signal (i.e. emission signal falling from F(i) into the filter used in the channel Ch(i)) to the total available signal (i.e. emission signal falling between the lasers before and after (i.e. L(i) and L(i+1)) into channel Ch(i)).*



Supplementary Figure S4. *Mean fluorescence intensity values obtained from droplet populations (P1 to P5) with different CB concentrations as acquired by MESA and CW modes. The error bars represent the standard deviation from mean and R is the correlation coefficient between the fluorescence intensities acquired by the two modes.*

Population	*FL conc. (μM)*	*MESA*		*CW*	
		Mean (μ)	*Standard deviation (σ)*	*Mean (μ)*	*Standard deviation (σ)*
P1	0.071	74.661	2.153	189.812	1.524
P2	0.214	103.181	1.828	196.327	1.647
P3	0.357	128.174	2.068	201.727	1.508
P4	0.500	153.848	2.240	209.024	1.732
P5	0.643	175.372	2.570	216.446	2.322

Supplementary Table S6. *Mean (μ) and (σ) standard deviations of FL fluorescence distribution of populations P1 – P5 obtained using CW and MESA mode. These values are used to calculate the Z-factor between the fluorescence distribution of separate droplet populations.*

First population (Pa)	*Second population (Pb)*	*Conc. Difference (μm)*	*Z-factor (MESA)*	*Z-factor (CW)*	*Mean Z-factor (MESA)*	*Std. Z-factor (MESA)*	*Mean Z-factor (CW)*	*Std. Z-factor (CW)*
P1	P2	0.143	0.581	-0.460	0.485	0.043	-0.546	0.148
P2	P3		0.532	-0.753				
P3	P4		0.497	-0.332				
P4	P5		0.330	-0.638				
P1	P3	0.286	0.763	0.237	0.743	0.032	0.219	0.017
P2	P4		0.759	0.202				
P3	P5		0.705	0.219				
P1	P4	0.429	0.834	0.492	0.825	0.012	0.450	0.059
P2	P5		0.817	0.408				
P1	P5	0.571	0.859	0.567	0.859	-	0.567	-

***Supplementary* Table S7.** Z-factors calculated using **Equation 3** between the droplet populations P1 – P5 separated by represented concentration differences.