Electronic Supplementary Material (ESI) for Lab on a Chip.

CD4+ versus CD8+ T-lymphocytes identification in integrated microfluidic chip using light scattering and machine learning

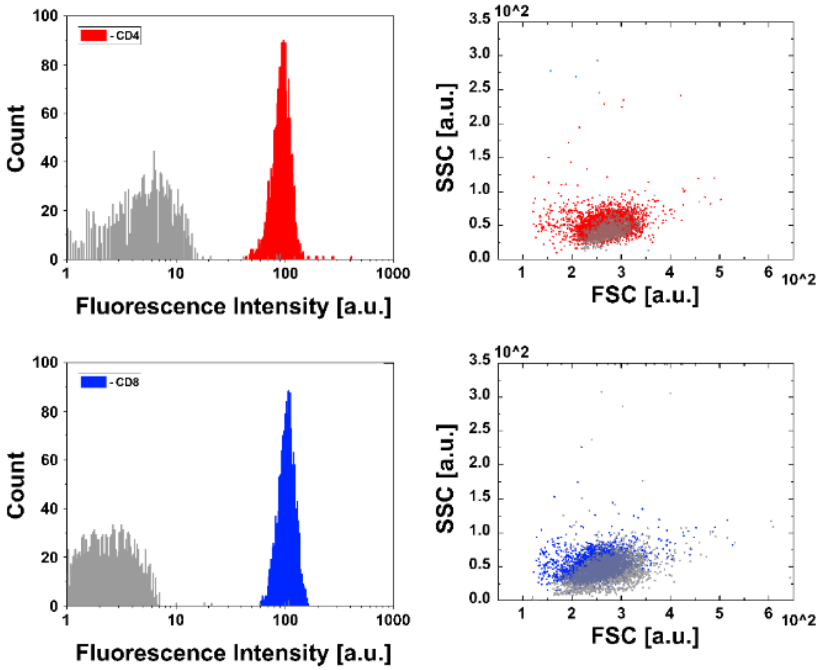
Domenico Rossi[1], David Dannhauser[1], Mariarosaria Telesco[1], Paolo A. Netti[1,2] & Filippo Causa[2]

[1] Center for Advanced Biomaterials for Healthcare@CRIB, Istituto Italiano di Tecnologia (IIT), Largo Barsanti e Matteucci 53, 80125 Naples, Italy.

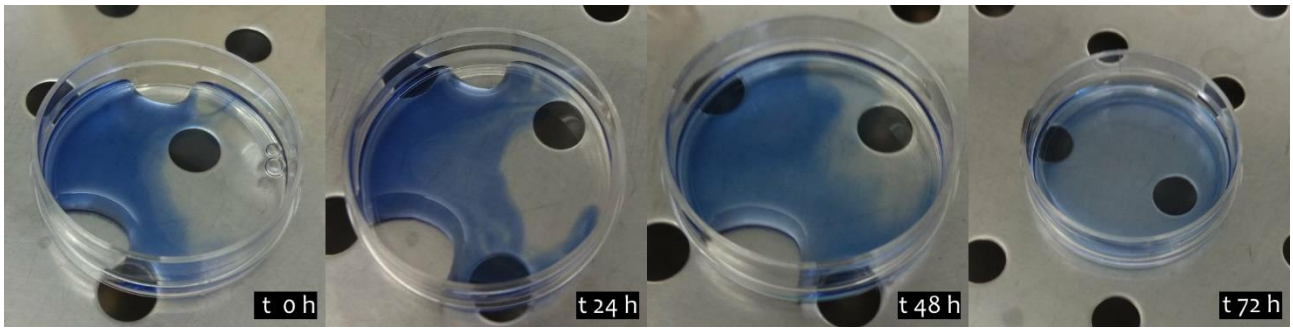
[2] Interdisciplinary Research Centre on Biomaterials (CRIB) and Dipartimento di Ingegneria Chimica, dei Materiali e della Produzione Industriale, Università degli Studi di Napoli "Federico II", Piazzale Tecchio 80, 80125 Naples, Italy.

*Correspondence to: causa@unina.it

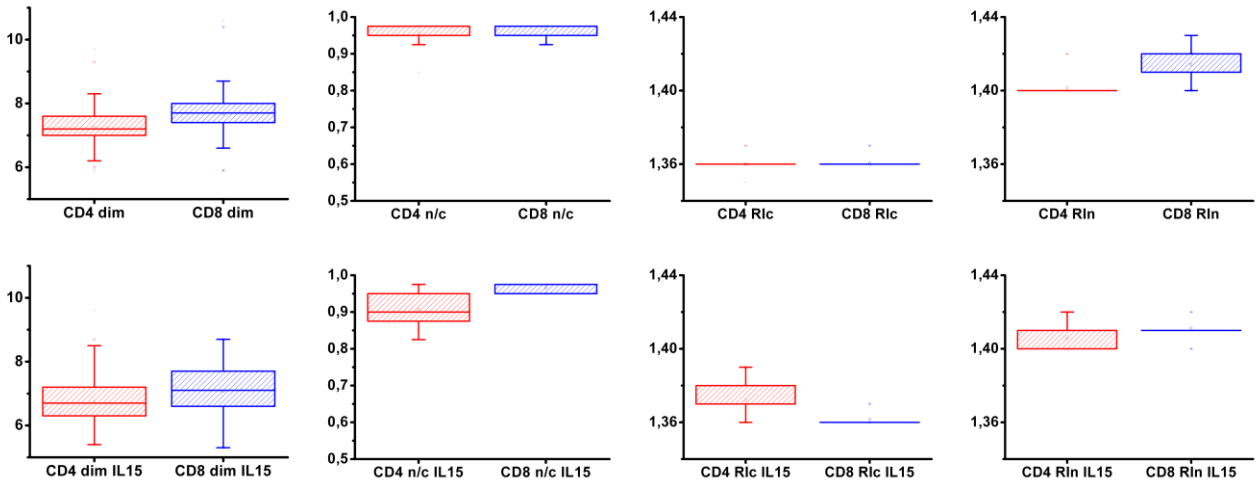
Supplementary information



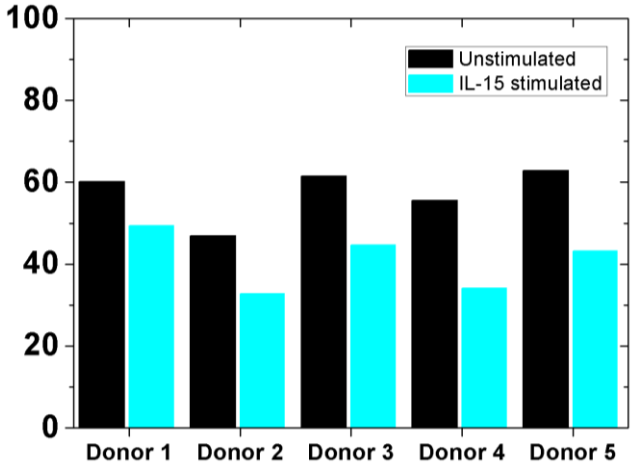
Supplementary fig. 1 Flow cytometer reference test have been performed to assure the purity of the T-lymphocytes subclasses isolation. A part of CD4+ and CD8+ was dedicated to this reference test and are not used for the subsequent Light Scattering experiments. Cells were investigated after isolation: on the right side the scatter plot (FSC vs SSC) is shown. The position of events (cells) on the scatterplot indicates CD4+ and CD8+ share the same area when investigated on the morphologic base alone: flow cytometer is not sensitive enough to discriminate them on morphologic base. On the left side fluorescence intensity indicating the degree of purity of the isolation is shown. Isolated CD4+ (upper line) were labeled with two Ab: Ab anti CD4 (red) showing positivity and Ab anti CD8+ (grey), showing negativity. Isolated CD8+ were labeled with two Ab: Ab anti Cd8+ (blue) showing positivity and Ab anti CD4 (grey) showing negativity. Please note that for both cell subclasses we performed two different runs with same instrument settings, and then we overlapped results.



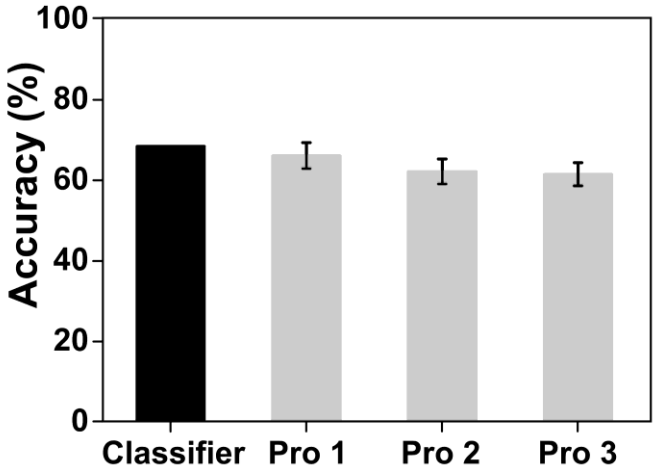
Supplementary fig. 2 Test for static mixing between water-based dye solution and viscoelastic alignment solution was performed. The two liquids were in contact, without any external force applied to enhance the mixing, and monitored over time. The complete mixing of the two liquids takes place at 72 h.



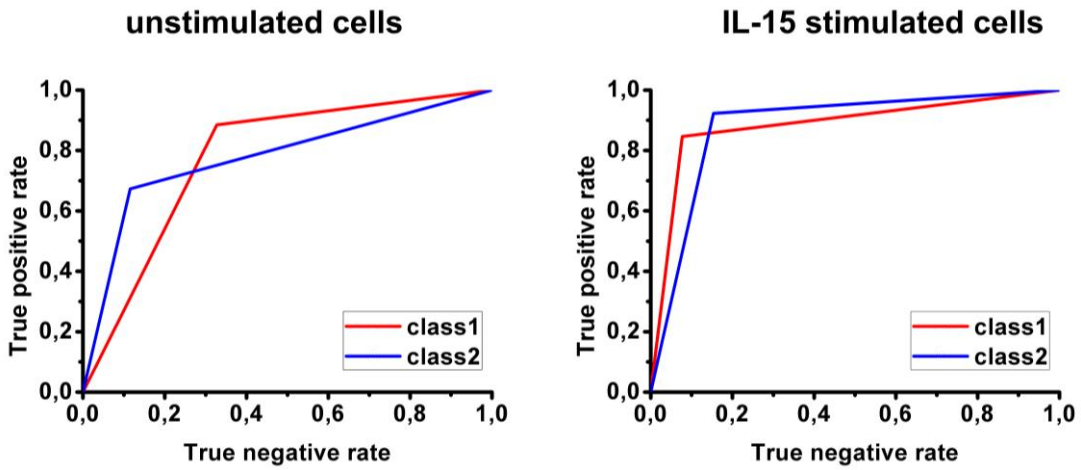
Supplementary fig. 3 Variations of biophysical properties of unstimulated vs stimulated CD4+ and CD8+. Cells were measured with blue laser. CD4+: red boxes, CD8+: blue boxes. Please note that the graph refers to values of biophysical properties of cell of donor 1: details on overlap of distributions of biophysical properties for each donor can be found in supplementary information, table 1.



Supplementary fig. 4 PDF overlapping for all donors (unstimulated versus IL-15 stimulated cells).



Supplementary fig. 5 Overall accuracy of ML approach performed on three different probands. The trained classifier for unstimulated CD4+ and CD8+ cells (red laser - indicated in black) was applied on different proband data and classifier accuracy was calculated. Results show similar outcomes for all probands compared to the used trained classifier. (Trained classifier: 68.49%; Pro 1: 66.13% ± 3.23; Pro 2: 62.21% ± 3.10; Pro 3: 61.52% ± 2.86).



Supplementary fig. 6 Receiver Operating Characteristic (ROC) curves of ML trainings, showing True positive rate vs True negative rate for both subclasses of T-lymphocytes (CD4+ and CD8+).

Supplementary table 1. Values of biophysical properties of CD4+ and CD8+ from all donors, together with values of PDF overlapping below.

	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	CD4+	CD8+	
	dimension (µm)		n/c ratio		RI_c		RI_n		
unstimulated cells									**average**
donor 1 (male, 33)	7.18 ± 0.60	7.51 ± 0.82	0.96 ± 0.02	0.96 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.01	
	73.50%		73.14%		71.59%		22.36%		60.15%
donor 2 (female, 35)	7.25 ± 0.76	7.13 ± 0.57	0.95 ± 0.02	0.97 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.01	
	84.89%		51.05%		37.51%		14.15%		46.90%
donor 3 (male, 35)	7.24 ± 0.67	7.06 ± 0.78	0.95 ± 0.02	0.97 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.00	
	87.73%		46.15%		100.00%		12.14%		61.50%
donor 4 (female, 27)	7.24 ± 0.67	7.17 ± 0.53	0.96 ± 0.01	0.97 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.01	
	87.13%		77.85%		81.38%		13.89%		55.56%
donor 5 (male, 36)	6.99 ± 0.54	7.20 ± 0.88	0.95 ± 0.02	0.97 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.40 ± 0.01	
	74.32%		49.39%		100.00%		27.59%		62.82%
average	7.18 ± 0.11	7.21 ± 0.18	0.95 ± 0.00	0.97 ± 0.00	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.00	
IL-15 stimulatd cells									**average**
donor 1 (male, 33)	7.29 ± 0.59	7.61 ± 0.67	0.95 ± 0.02	0.97 ± 0.01	1.36 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.01	
	81.25%		19.49%		34.13%		62.41%		49.43%
donor 2 (female, 35)	7.22 ± 0.66	7.38 ± 0.82	0.95 ± 0.01	0.97 ± 0.01	1.37 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.00	
	86.34%		36.78%		0.54%		7.28%		32.74%
donor 3 (male, 35)	7.32 ± 0.75	7.41 ± 0.68	0.95 ± 0.02	0.96 ± 0.01	1.37 ± 0.00	1.36 ± 0.00	1.40 ± 0.01	1.41 ± 0.01	
	93.20%		53.37%		7.80%		20.33%		44.68%
donor 4 (female, 27)	7.44 ± 0.83	7.34 ± 0.75	0.95 ± 0.01	0.97 ± 0.01	1.37 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.01	
	92.99%		32.44%		4.90%		6.22%		34.14%
donor 5 (male, 36)	7.40 ± 1.14	7.65 ± 1.07	0.95 ± 0.02	0.96 ± 0.02	1.37 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.00	
	89.33%		56.33%		15.39%		11.92%		43.24%
average	7.33 ± 0.09	7.48 ± 0.14	0.95 ± 0.00	0.97 ± 0.01	1.37 ± 0.00	1.36 ± 0.00	1.40 ± 0.00	1.41 ± 0.00	

Supplementary table 2. Comparison between ML trained classifier outcomes of unstimulated cells with red versus blue laser.

Red laser - training			
	CD4+	**CD8+**	**precision**
CD4+	248	16	93.94%
CD8+	151	115	43.23%
	sensitivity	**specificity**	**accuracy**
	62.16%	87.79%	**68.49%**

Blue laser - training			
	CD4+	**CD8+**	**precision**
CD4+	483	63	88.46%
CD8+	144	296	67.27%
	sensitivity	**specificity**	**accuracy**
	77.03%	82.45%	**79.01%**

Supplementary table 3. Results of ML on unstimulated and stimulated cells for all donors.

unstimulated cells			
training			
CD4+	483	63	88.46%
CD8+	144	296	67.27%
	sensitivity	**specificity**	**accuracy**
	77.03%	82.45%	79.01%

test donor 1			
CD4+	106	36	74.56%
CD8+	77	104	57.46%
	sensitivity	**specificity**	**accuracy**
	57.92%	74.29%	65.02%

test donor 2			
CD4+	77	43	64.17%
CD8+	21	70	76.92%
	sensitivity	**specificity**	**accuracy**
	78.57%	61.95%	69.97%

test donor 3			
CD4+	76	8	74.16%
CD8+	13	54	79.03%
	sensitivity	**specificity**	**accuracy**
	85.54%	68.06%	76.16%

test donor 4			
CD4+	115	6	82.08%
CD8+	25	76	54.31%
	sensitivity	**specificity**	**accuracy**
	62.14%	76.83%	67.57%

test donor 5			
CD4+	129	11	92.14%
CD8+	55	48	46.60%
	sensitivity	**specificity**	**accuracy**
	70.11%	81.36%	72.84%

stimulated cells			
training			
CD4+	176	32	84.62%
CD8+	13	155	92.26%
	sensitivity	**specificity**	**accuracy**
	93.12%	82.89%	88.03%

test donor 1			
CD4+	38	12	76.00%
CD8+	7	54	88.52%
	sensitivity	**specificity**	**accuracy**
	84.44%	81.82%	82.88%

test donor 2			
CD4+	96	4	69.00%
CD8+	15	58	79.45%
	sensitivity	**specificity**	**accuracy**
	86.49%	93.55%	89.02%

test donor 3			
CD4+	115	25	82.14%
CD8+	21	78	78.79%
	sensitivity	**specificity**	**accuracy**
	84.56%	75.73%	80.75%

test donor 4			
CD4+	84	3	96.55%
CD8+	17	105	86.07%
	sensitivity	**specificity**	**accuracy**
	83.17%	97.22%	90.43%

test donor 5			
CD4+	56	11	83.58%
CD8+	5	52	91.23%
	sensitivity	**specificity**	**accuracy**
	91.80%	82.54%	87.10%

Supplementary table 4. Comparison between ML trained classifier outcomes with blue laser with IL-15 stimulated cells at three different times: 0 minutes (NO IL-15), 30 minutes and 240 minutes.

0 minutes (NO IL-15) - training				30 minutes - training				240 minutes - training			
	CD4+	CD8+	precision		CD4+	CD8+	precision		CD4+	CD8+	precision
CD4+	483	63	88.46%	CD4+	176	32	84.62%	CD4+	372	37	90.95%
CD8+	144	296	82.45%	CD8+	13	155	92.89%	CD8+	45	453	89.12%
	sensitivity	specificity	accuracy		sensitivity	specificity	accuracy		sensitivity	specificity	accuracy
	77.03%	82.45%	**79.01%**		93.12%	82.89%	**88.03%**		82.91%	92.45%	**90.96%**

Supplementary table 5. ML results on mixed samples for each donor.

	cells (Neubauer chamber)		CV (%)	MIX A (CD4+/CD8+ = 2.5)	MIX C (CD4+/CD8+ = 1.0)	MIX B (CD4+/CD8+ = 0.5)	MIX A (CD4+/CD8+ = 2.5)	MIX C (CD4+/CD8+ = 1.0)	MIX B (CD4+/CD8+ = 0.5)
				unstimulated cells			IL-15 stimulated cells		
	CD4+	CD8+		ML result			ML result		
donor 1	**191**	**163**	5.3%	1.68 ± 0.13 p = 0.005	0.67 ± 0.04 p = 0.02	0.48 ± 0.12 p = 0.78	2.35 ± 0.08 p = 0.3	1.02 ± 0.07 p = 0.9	0.49 ± 0.01 p = 0.7
donor 2	**184**	**172**	5.3%	3.10 ± 0.05 p = 0.01	0.86 ± 0.07 p = 0.01	1.58 ± 0.23 p = 0.03	2.65 ± 0.34 p = 0.33	0.55 ± 0.09 p = 0.32	1.00 ± 0.09 p = 0.86
donor 3	**149**	**117**	6.1%	1.95 ± 0.04 p = 0.01	0.68 ± 0.09 p = 0.03	1.06 ± 0.19 p = 0.52	2.17 ± 0.35 p = 0.1	0.45 ± 0.1 p = 0.37	0.90 ± 0.13 p = 0.17
donor 4	**216**	**205**	4.9%	2.11 ± 0.42 p = 0.15	0.90 ± 0.21 p = 0.21	1.53 ± 0.12 p = 0.005	2.77 ± 0.29 p = 0.10	0.50 ± 0.02 P = 0.72	1.00 ± 0.11 p = 0.98
donor 5	**104**	**98**	7.0%	2.78 ± 0.54 p = 0.32	1.08 ± 0.20 p = 0.03	1.25 ± 0.29 p = 0.22	2.16 ± 1.00 p = 0.55	0.56 ± 0.18 p = 0.54	0.96 ± 0.23 p = 0.71