A Method for Detecting Protein Expression in Single Cells Using the C_1™ Single-Cell Auto Prep System

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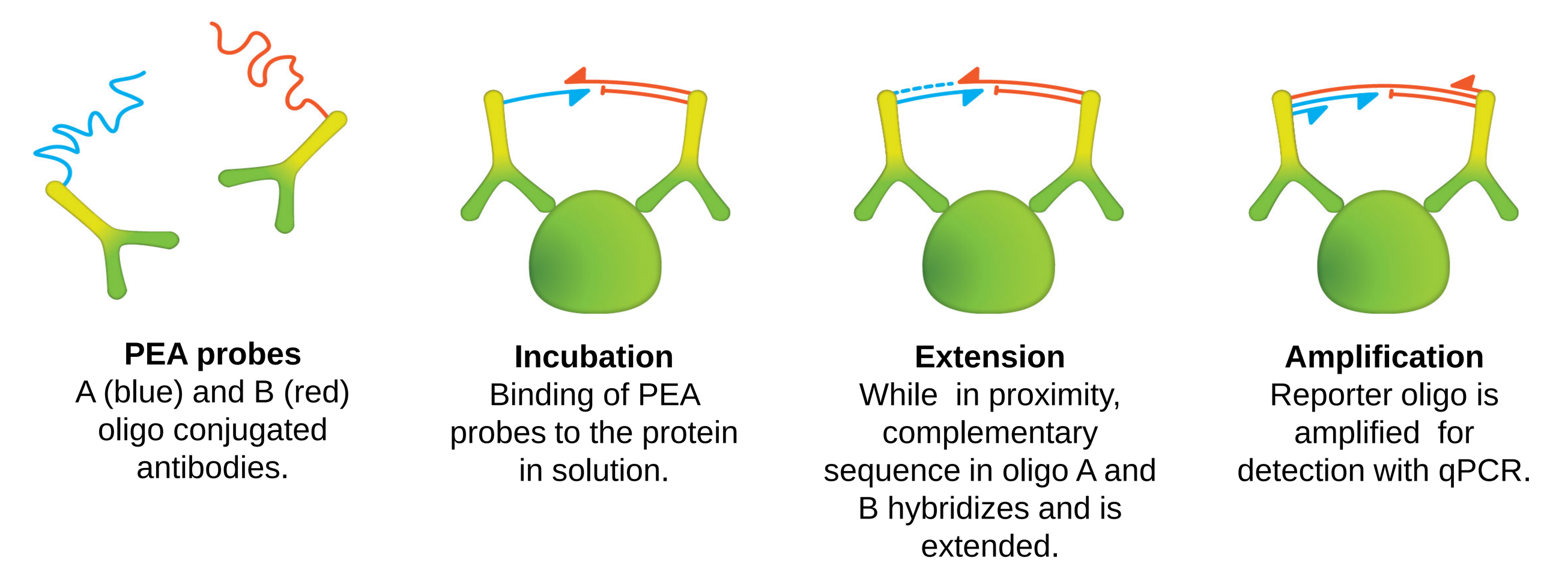
Introduction

Recent improvements in microfluidics and biochemistry have enabled single-cell molecular analysis, providing new insight into the heterogeneity of cell populations. The C_1™ Single-Cell Auto Prep System is an automated platform that streamlines the isolation and processing of 96 individual, live cells for RNA and DNA analysis. Single-cell protein profiling is a direct complement to genomic analysis as it provides additional insights into key molecular mechanisms and system biology. To enable this, we adapted a highly multiplexed protein detection method (Proseek Multiplex Oncology I ^{96x96}, Olink Bioscience) based on the Proximity Extension Assay technology (PEA) for use on the C_1™ Single-Cell Auto Prep System.

Overview of the C_1™ Single-Cell Auto Prep System for Protein Detection

We have used the C_1™ Single-Cell Auto Prep System in combination with the Proximity Extension Assay technology (PEA, Figure 1A) to develop a workflow for the automated analysis of the protein expression of single cells (Figure 1B-D). The method developed is based on the use of a PEA probe panel targeting 92 different proteins and of those 66 correspond to intracellular proteins that can be detected in single cells (Figure 1E).

A



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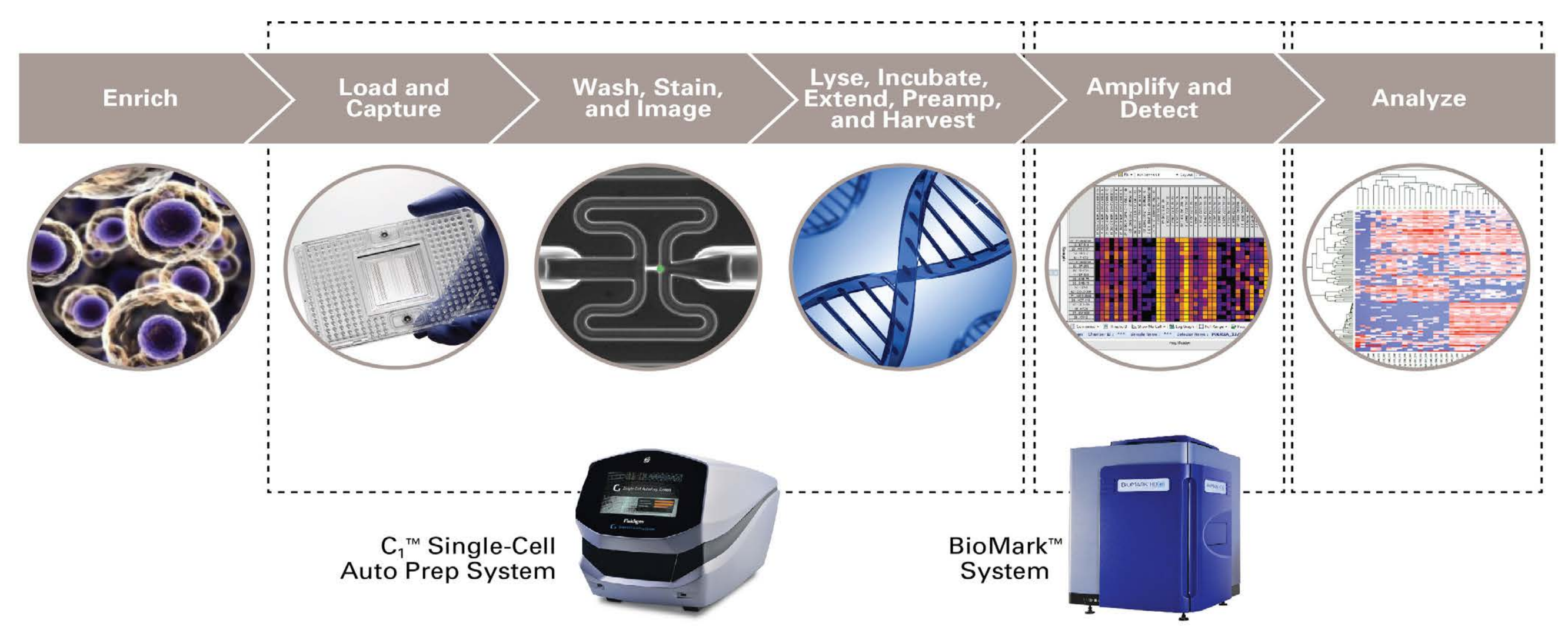

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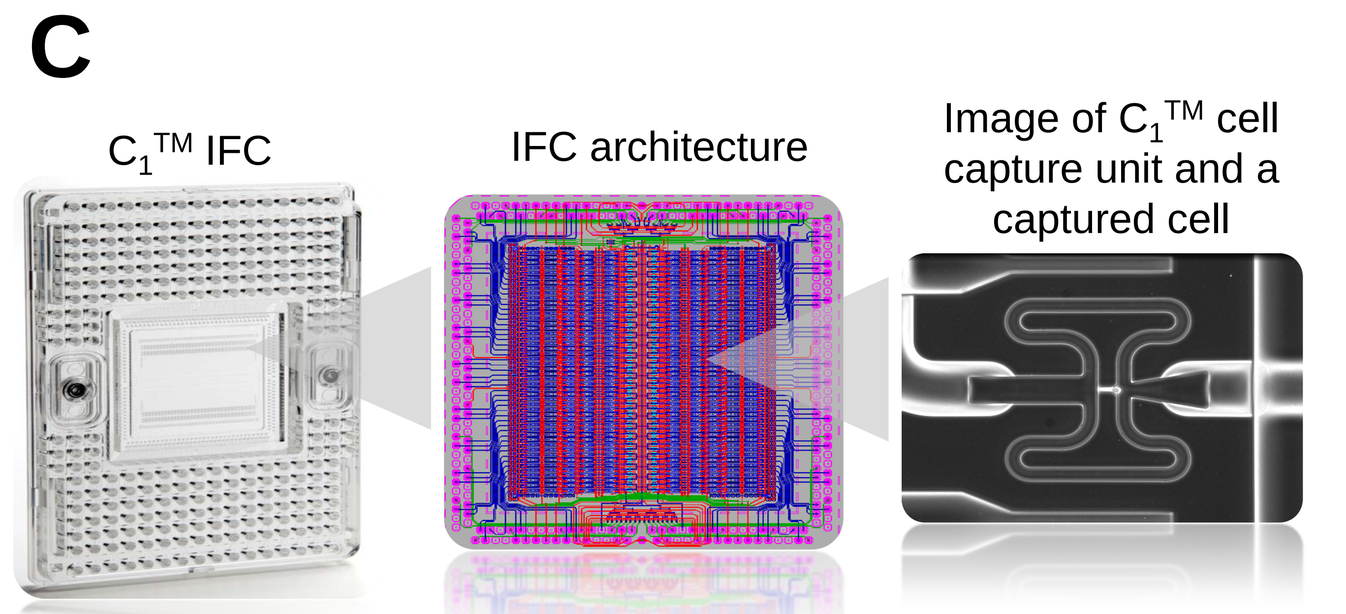

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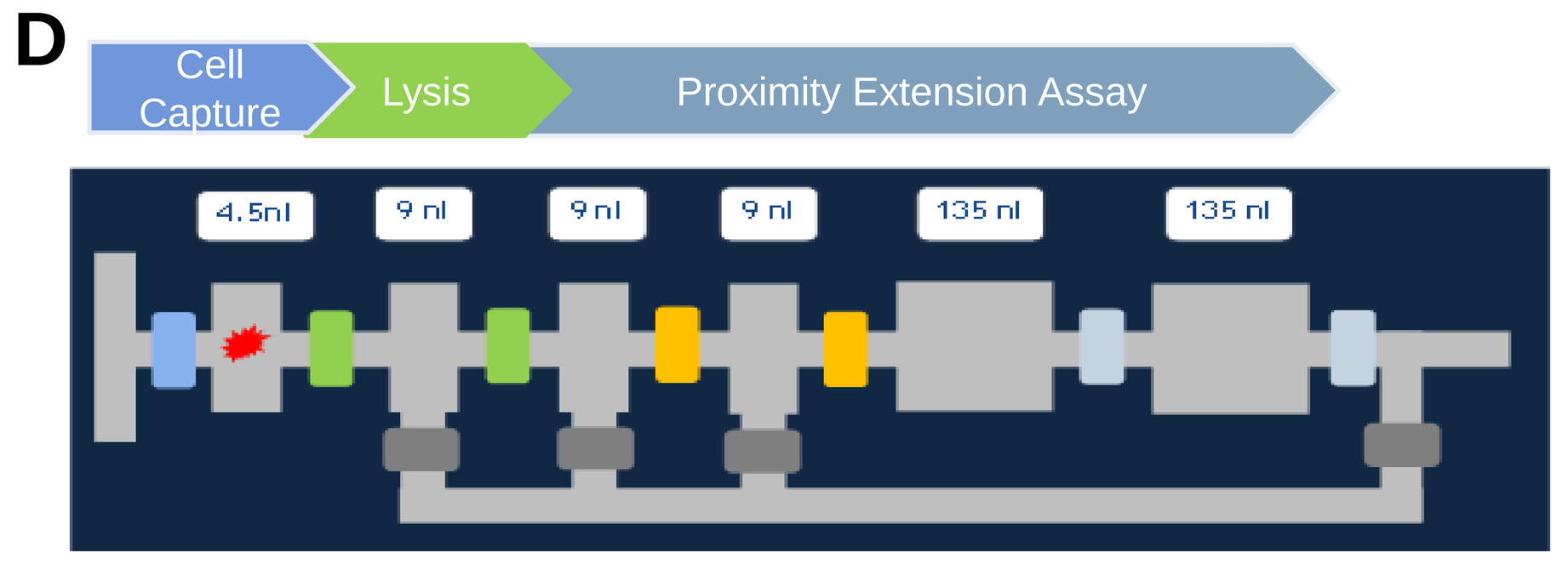


E

Assay ID	Target	Assay ID	Target	Assay ID	Target	Assay ID	Target	Assay ID	Target	Assay ID	Target
101	IL-8	117	Galectin-3	133	CXCL9	149	EMMPRIN	165	Betacellulin	181	VEGF-D
102	VEGF	118	Erythropoietin	134	IL-6 R alpha	150	Carbonic Anhydrase IX	166	Epiregulin	182	HE4
103	Adrenomedullin	119	Extension control	135	TNF RII	151	CXCL10	167	Flt-3 Ligand	183	CXCL13
104	GFP control	120	TGFb1	136	MMP-3	152	EpCAM	168	VEGF R2	184	EGFR
105	CD40L	121	Kallikrein 11	137	IL-2 R alpha	153	ErbB2	169	CCL21	185	HGF R
106	GDF15	122	Tie-2	138	LIGHT	154	ErbB3	170	Caspase-3	186	Thrombopoietin
107	PIGF	123	Tissue Factor	139	Prolactin	155	ErbB4	171	CD69	187	FABP4
108	E-selectin	124	TNF RI	140	Myeloperoxidase	156	HGF	172	OX40	188	CEACAM-5
109	EGF	125	PDGF-BB	141	Growth Hormone	157	Kallikrein 3	173	TRACP	189	TNF alpha
110	Osteoprotegerin	126	GM-CSF	142	Fas Ligand	158	MyD88	174	CD30L	190	CA242
111	IFNg	127	M-CSF	143	BAFF	159	MIA	175	Reg4	191	CA125
112	IL-1ra	128	CXCL11	144	Fas	160	CCL24	176	TGF-alpha	192	Prostasin
113	IL-6	129	IL-12	145	CCL19	161	Midkine	177	Amphiregulin	193	Follistatin
114	Cystatin B	130	IL-2	146	PE control	162	uPAR	178	HB-EGF	194	CD31
115	MCP-1	131	IL-7	147	Oligo-Ref control	163	CXCL5	179	MICA	195	IL17BR
116	Kallikrein 6	132	SCF	148	ER alpha	164	Cathepsin D	180	IL-4	196	FOLR1

F

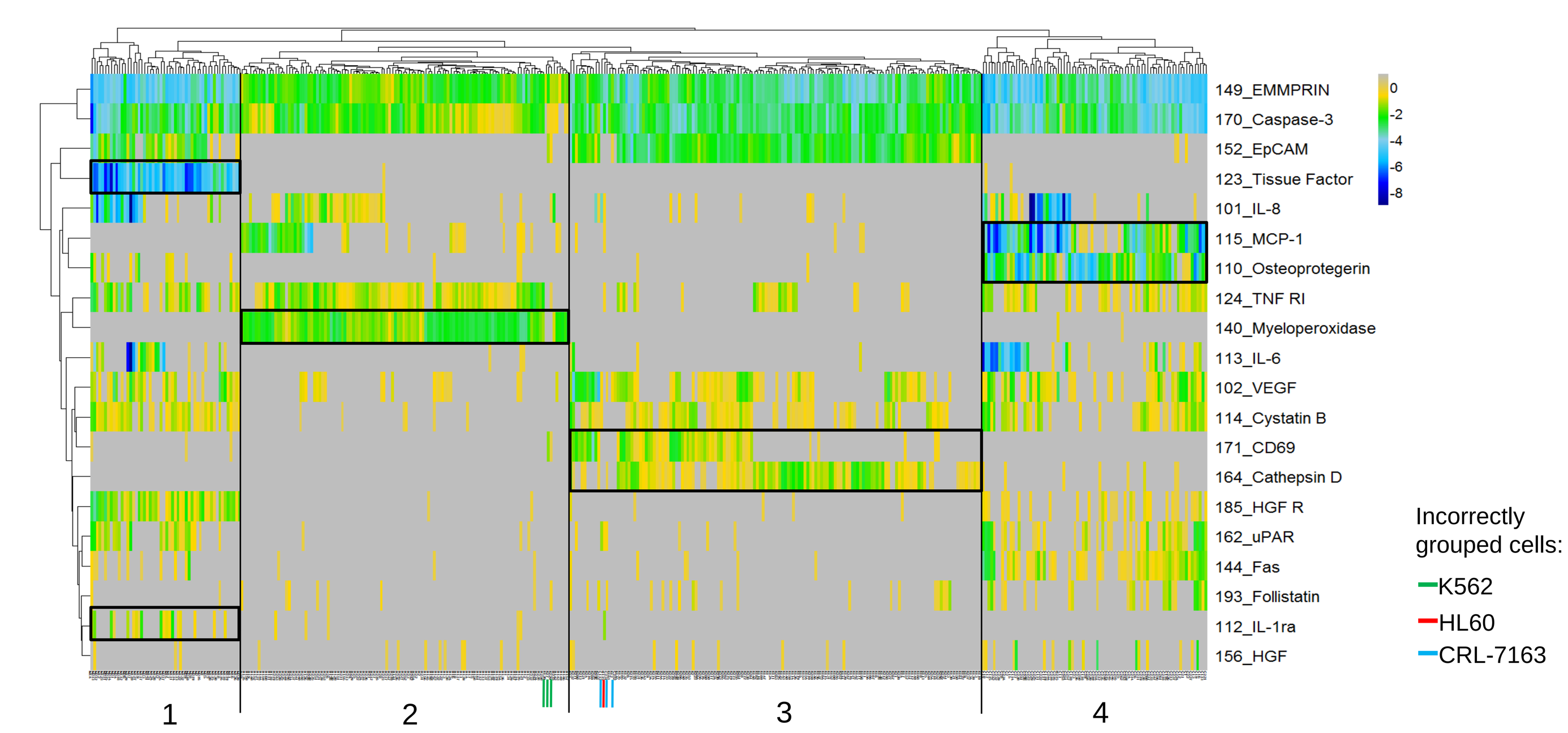
Process	Total Time to Complete	Hands-on Time
C_1™ Proximity Extension Assay	8 hrs	1.5 hrs
BioMark™ System	4 hrs	1.5 hrs

Figure 1

A: Schematic representation of the PEA method. Detection of the amplified reporter oligonucleotide is done by qPCR on the BioMark™ System. Cycle threshold of the amplified reporter oligo reflects target protein abundance during the incubation step. **B**: The workflow developed for automated protein detection in single cell uses the C_1™ Single-Cell Auto Prep System that is composed of a controller instrument and integrated fluidic circuits (IFC). **C**: The C_1™ IFC architecture is shown in details, containing 96 individual capture sites and dedicated nano-chambers for downstream reactions. **D**: Representation of the system of independent chambers and valves connected to the 4.5 nL single-cell capture site in the C_1™ IFC. Each one of the 96 capture sites has its own dedicated system of chambers and valves, allowing all PEA steps to take place in a single run for 96 single cells in parallel. **C**: List of protein targets for the PEA probe panel contained in the Proseek Multiplex Oncology I ^{96x96} kit used. Of the 92 protein targets, 25 (around 30%) are strictly secreted and not expected to generate signal when performing single cell analysis. **D**: Single-cell-to-result turnaround time.

Results

Characteristic Protein Expression Signatures Identified



Cell lines used:

1. MDA-MB-231: epithelial cells originated from metastatic sites of individuals with breast adenocarcinoma
2. HL60: promyeloblasts from peripheral blood of individuals with acute promyelocytic leukemia
3. K562: lymphoblasts from the bone marrow of individuals with chronic myelogenous leukemia
4. CRL-7163: fibroblasts from the thymus

Figure 3

A total of 401 single cells were analyzed (represented in columns in the panel above) in eight independent C_1™ PEA experiments for each of the four human cell lines MDA-MB-231 (n=54), CRL-7163 (n=83), HL60 (n=117), and K562 (n=147) (ATCC). Protein targets are represented in horizontal lines in the panel above. Across the two experiments run for each cell line, 41, 31, 24, and 56 protein targets were detected as expressed in at least one single cell, respectively. Protein targets are considered expressed if ΔC_T = Sample C_T - (Avg. Background C_T - 2*St. Dev. Background) < -0.4. The figure shows targets detected as expressed in a minimum of 10% of all single cells within each cell line analyzed. Of the 20 targets shown in the figure above, seven stand out as having somewhat specific expression levels in the following cell lines: Tissue Factor and IL-1ra in MDA-MB-231; Myeloperoxidase in HL60; CD69 and Cathepsin D in K562; MCP-1 and Osteoprotegerin in CRL-7163. Expression in specific cell lines and corresponding specific function was validated by literature analysis (References).

Most Targets Detected in Single Cells are Consistently Detected Across Experiments

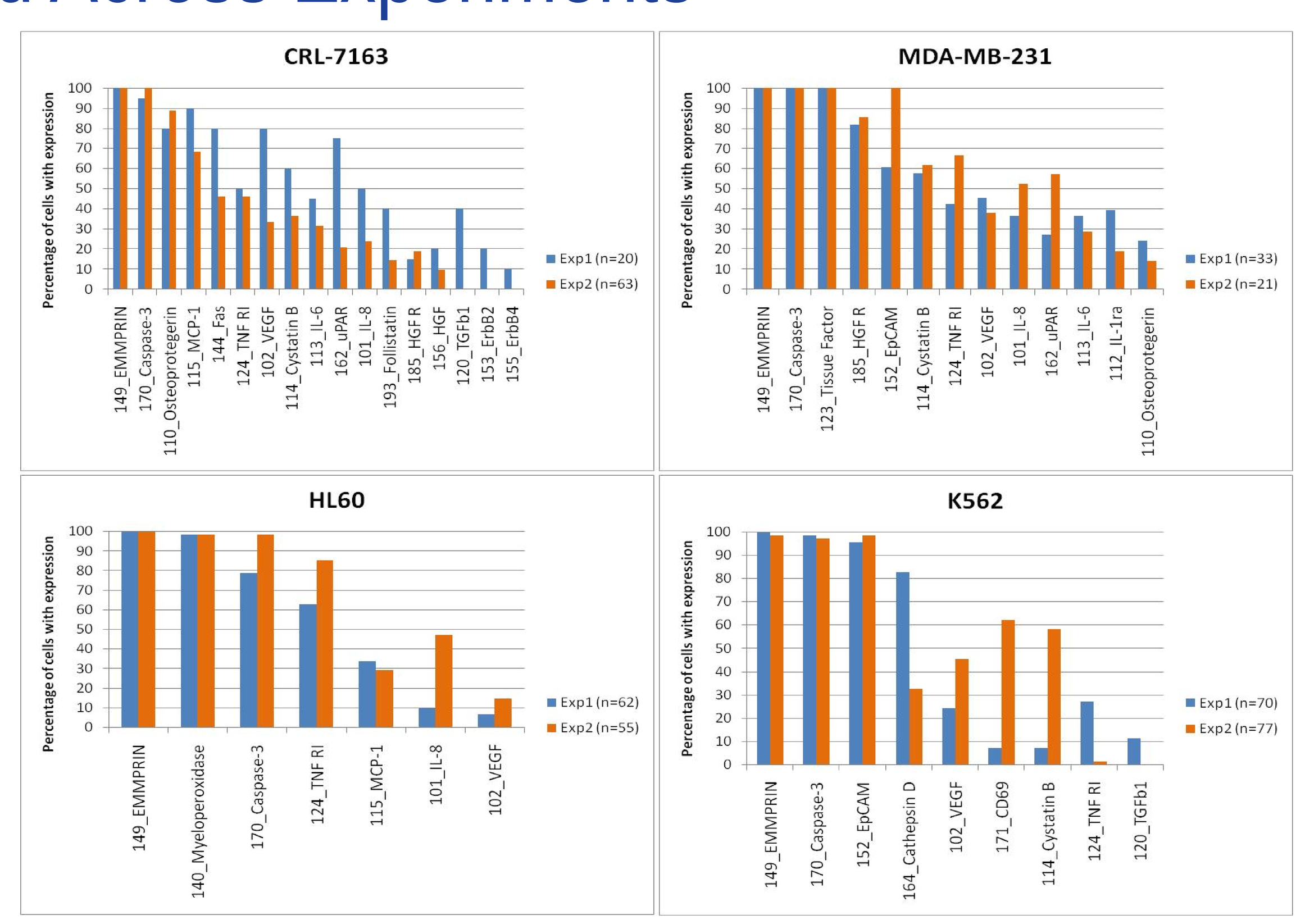


Figure 4

The graphs above show targets detected in specific cell lines tested across two independent C_1™ PEA experiments. Since some level of variability of protein expression is expected at single-cell level, a more stringent criteria was used to select top targets expressed in the cell lines to evaluate experimental reproducibility: targets expressed in at least 10% of all single cells within at least one experiment with ΔC_T = Sample C_T - (Avg. Background C_T - 2*St. Dev. Background) < - 0.4 are shown. On average, 90% of the targets shown for each cell line were consistently expressed across the two experimental replicates at similar percentages of the cell population analyzed.

Protein Detection in Single Cells is Consistent Across Chip and Plate Experiments

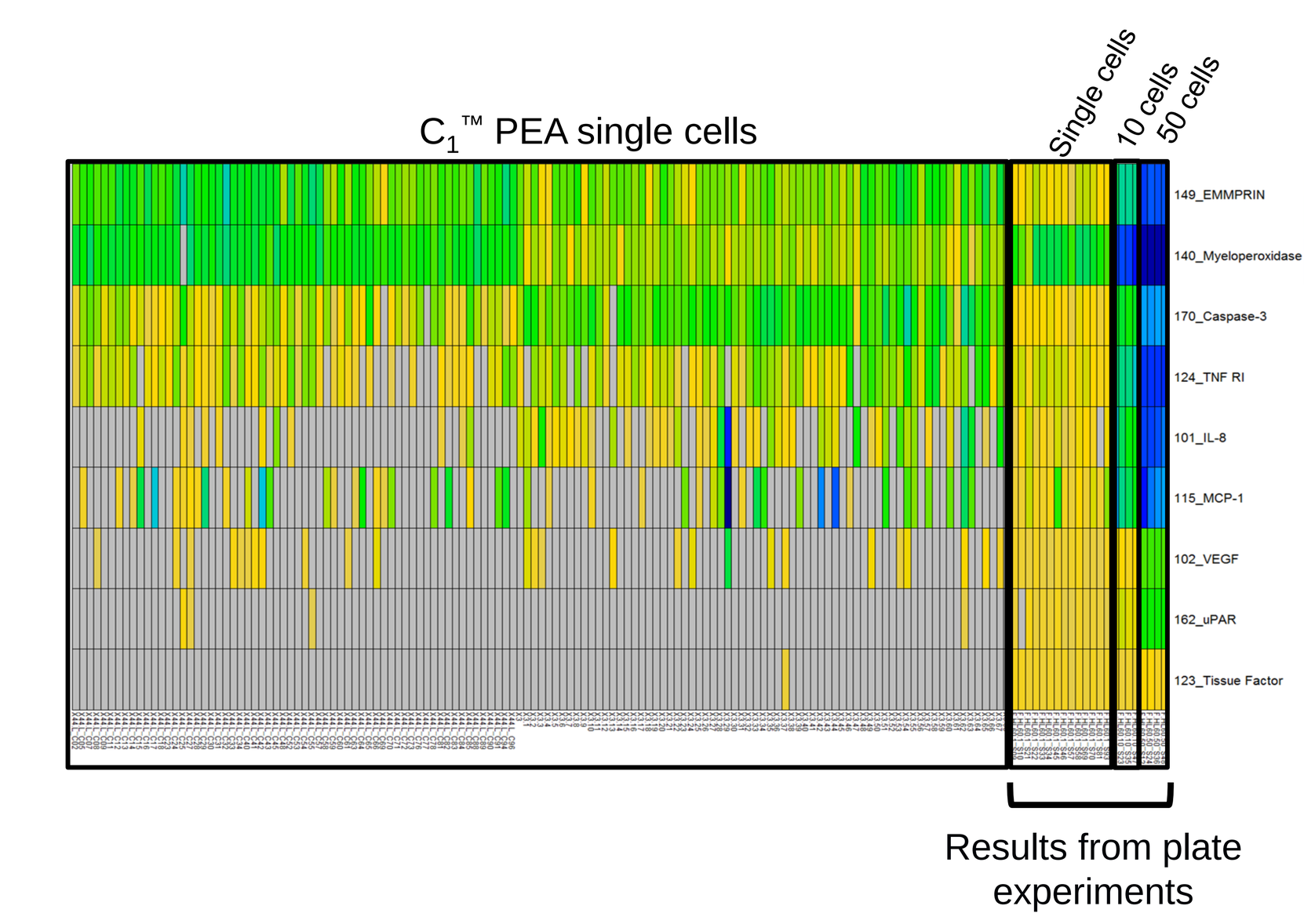


Figure 5

Results from PEA on plate-sorted cells were compared to results obtained from two independent C_1™ PEA experiments on single HL60 cells. In general, results obtained from plate PEA on sorted cells confirmed results obtained by C_1™ PEA, with the exception of Tissue Factor. However, plate PEA signal for this specific target does not increase as expected when 10 and 50 cells are tested, suggesting that the high background signal of plate PEA could be affecting expression level results for this method.

Flow Cytometry and Immunofluorescence Results are Consistent with C_1™ PEA Results

A

Cell type	K562			HL60		
Analysis method	PEA (n=22)	Flow (n=2000)	IF (n=22)	PEA (n=15)	Flow (n=2000)	IF (n=15)
EpCAM	86%	100%	100%	3%	0.3%	7%
EMMPRIN	100%	100%	NA	100%	100%	100%

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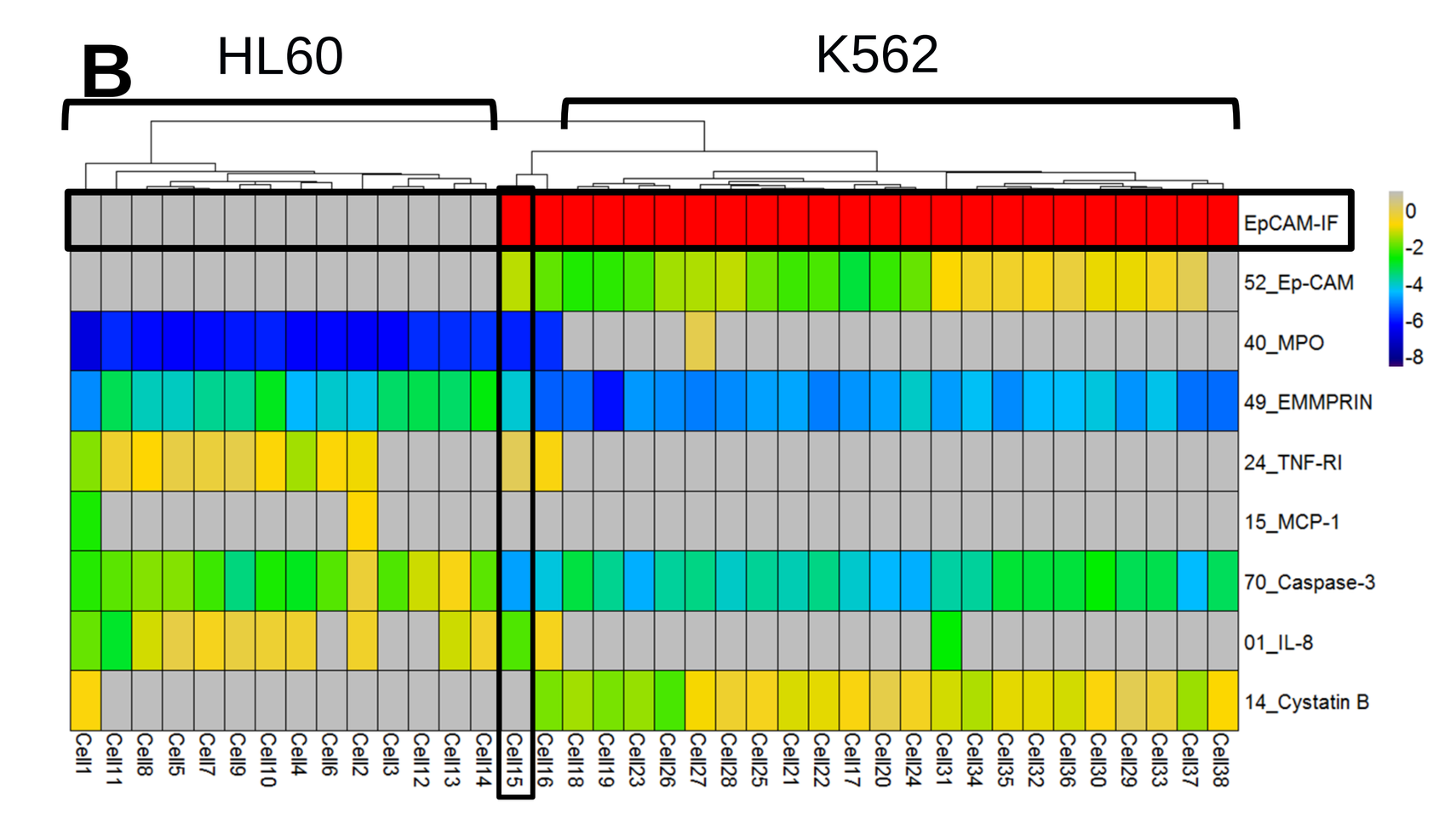

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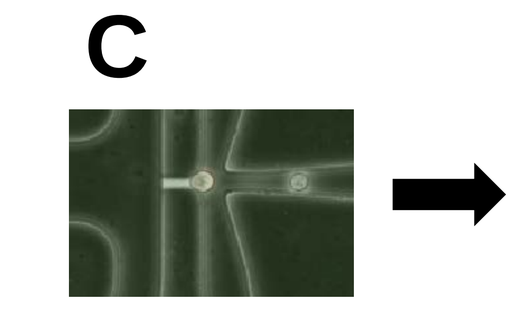

Two cell types captured in the same chamber could explain signal detected for both EpCAM via IF and PEA (specific to K562) and MPO via PEA (specific to HL60) for the same sample.

Figure 6

A: C_1™ PEA results for two specific targets were validated on HL60 and K562 cells using orthogonal methods: EpCAM (low and high expression, respectively) and EMMPRIN (high expression in both cell types) antibodies conjugated with fluorescent dyes were used to evaluate expression levels of populations of cells with flow cytometry (Flow) and for on-chip immunofluorescence (IF) on single cells prior to C_1™ PEA. Flow and IF results were highly concordant with PEA results and some of the expression rate differences observed can be explained by different antibodies used across the methods and different population of cells tested (flow vs. PEA and IF). **B**: The diagram shows a heat map of the protein expression results for C_1™ PEA and IF for EpCAM (red indicates high expression). As expected, K562 cells have high EpCAM expression confirmed by PEA and IF and HL60 cells have high MPO expression levels confirmed by PEA. Two cells out of 38 analyzed with IF and PEA had results different than expected, presenting both EpCAM expression (IF and PEA) and MPO (PEA). For one of those cells it has been confirmed that two instead of one cell had been captured in the C_1™ IFC chamber (panel **C**).

Conclusion

- We have developed methodology for automated protein detection from single cells on the C_1™ Single-Cell Auto Prep System, with the ability to simultaneously process up to 96 single cells.
- The method is sensitive enough to detect expression levels from single cells and is a promising technique to use in combination with DNA and RNA profiling from single cells for further system biology studies. It is also consistent with other studies that target gene expression (References).
- The PEA probe panel from the Proseek Multiplex Oncology I ^{96x96} kit, which targets 92 potential cancer-related targets, has been successfully used in profiling single cells derived from both cancer and normal tissue, grouping 98% of all cells analyzed (n=401).

References

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