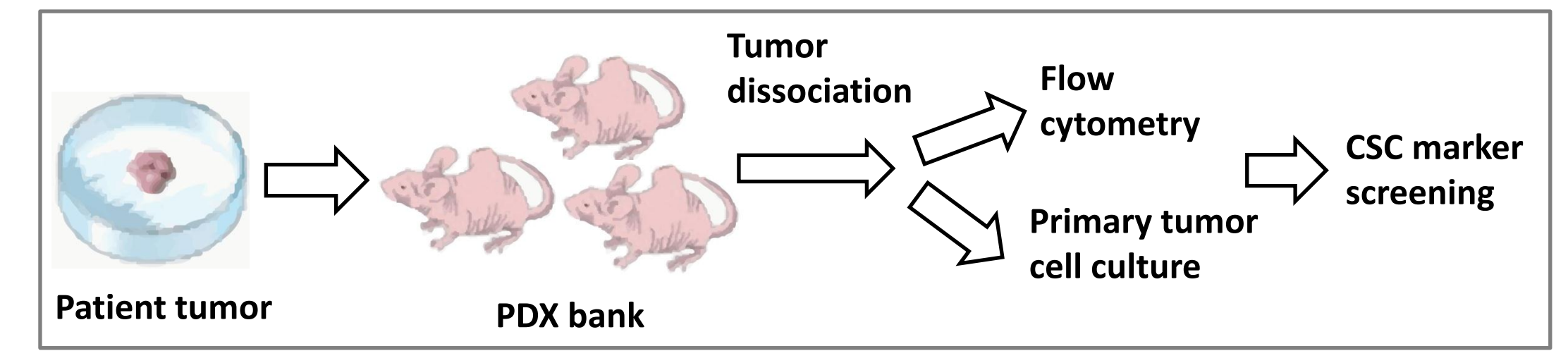


Andrea Aloia<sup>1</sup>, Evgeniya Petrova<sup>2</sup>, Olivier Deas<sup>3</sup>, Sophie Banis<sup>3</sup>, Enora Le Ven<sup>3</sup>, Andreas Bosio<sup>1</sup>, Olaf Hardt<sup>1</sup>, Stefano Cairo<sup>3</sup>, Jean-Gabriel Judde<sup>3</sup>. <sup>1</sup>Miltenyi Biotec GmbH, Bergisch Gladbach, Germany; <sup>2</sup>Institut Pasteur, Paris, France; <sup>3</sup>Xentech, Evry, France

Tumor recurrence fueled by residual tumor cells having survived chemotherapy represents the principal cause of breast cancer treatment failure. Triple-negative breast cancer (TNBC) is a heterogeneous disease at both molecular and cellular level, and the presence of different tumor cell sub-populations is likely the reason for this heterogeneity and for the incomplete response to neoadjuvant chemotherapy observed for most TNBCs. To identify and isolate tumor cell sub-populations that resist to chemotherapy, we used a panel of 45 antibody-fluorochrome conjugates in combination with multi-parameter flow cytometry to screen for the expression of a set of cell surface markers in residual tumor cells that survive chemotherapy. This set of markers represented both proteins involved in stem cell function and proteins known to be over-expressed in stem cells or cancer stem cell sub-populations. As a source of tumor samples, we used a panel of TNBC patient-derived xenografts (PDXs). These tumor models are known to preserve the morphology, molecular characteristics and drug response profile of the original patient tumors. We used TNBC PDX models to reproduce *in vivo* chemotherapy-induced tumor regression and relapse.

### Searching for the best cancer stem cell marker: problem and solution

#### PDX tumor bank as a source of human breast cancer tissue



Currently available CSC markers have heterogeneous expression in tumors of the same type and cannot predict tumor response to standard chemotherapy

Model	Origin	Type	% CSC marker-positive cells			Drug response		
			CD44 <sup>+</sup> CD24 <sup>low</sup>	ALDH <sup>high</sup>	CD133 <sup>+</sup>	Docetaxel	Capecitabine	Doxorubicin/Cyclophosphamide
HBCx-2	Metastasis	TN	0.3	0.8	0	NR	NR	NR
HBCx-3	Primary	ER+	0.1	5	0	NR	NR	NR
HBCx-5	Primary	HER2+	0	16	0	NR	HR	NR
HBCx-6	Primary	TN	0.1	3	79	NR	R	HR
HBCx-7	Metastasis	TN	1.5	5	0	R	R	NR
HBCx-9	Primary	TN	27	5	29	R	R	R
HBCx-10	Primary	TN	0	0.5	3.7	NR	R	HR
HBCx-11	Primary	TN	0.3	2.7	0.4	NR	R	R
HBCx-12B	Metastasis	TN	11	8.3	76	NR	R	LR
HBCx-14	Primary	TN	0	32	67	NR	R	HR
HBCx-15	Primary	TN	65	5	0	R	R	HR
HBCx-16	Primary	TN	24	0.1	16	R	HR	NR
HBCx-17	Primary	TN	3	48	61	NR	HR	HR
HBCx-19	Metastasis	ER+	82	22	29	NR	NR	NR
HBCx-24	Primary	TN, EGFR+	11	50	73	R	R	NR
HBCx-27	Primary	TN	0	38	50	NA	NA	NA
HBCx-28	Primary	TN	6	14	51	LR	NR	R
HBCx-33	Primary	TN	20	59	50	NR	R	HR
HBCx-34	Primary	ER+PR+HER2-	7.7	33	0.7	R	R	HR
HBCx-39	Primary	TN	7	40	20	NR	HR	LR
T168	Primary	ER+PR+HER2-	54	52	66	R	HR	HR
T30	Primary	TN	-	1.5	11	NR	NR	HR

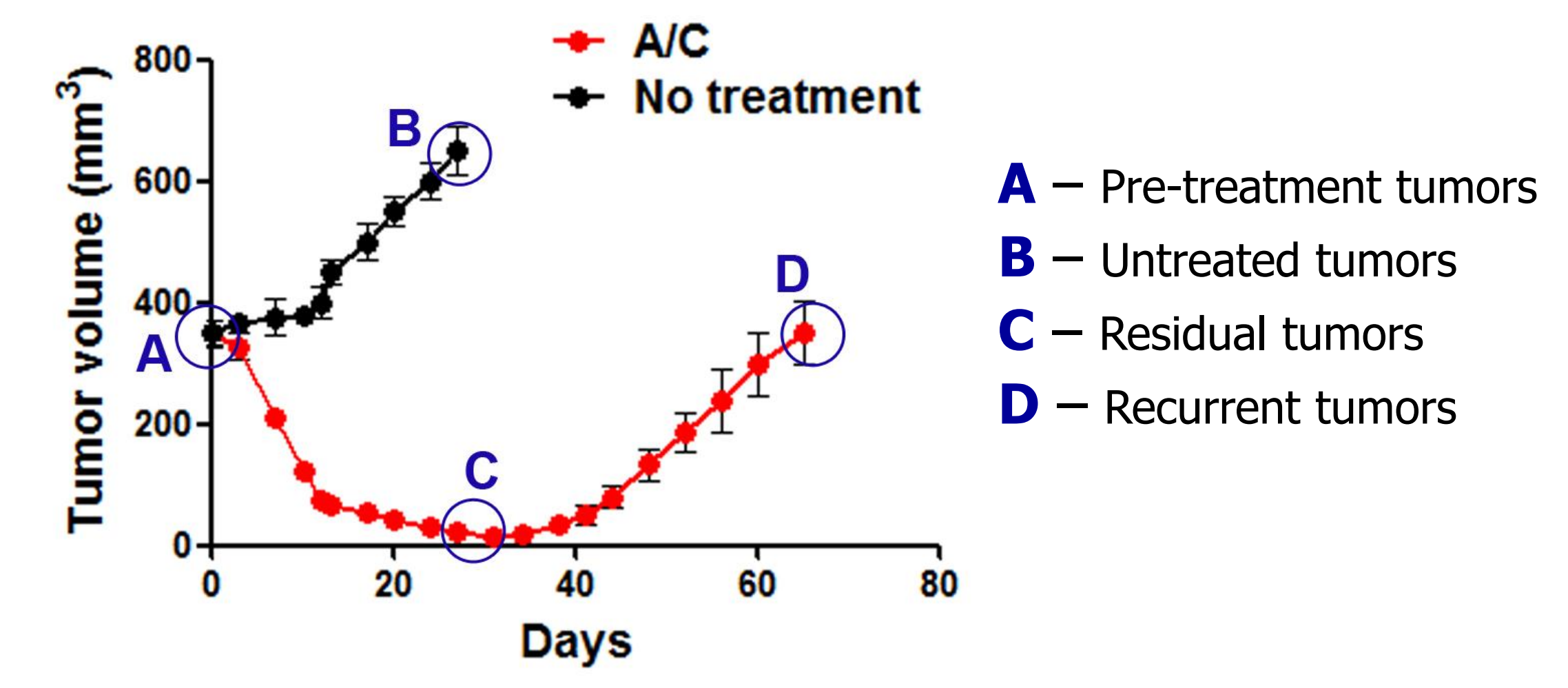
There is a need for better markers of chemotherapy-resistant cells / CSCs

#### Cell surface marker antibody library: a tool to search for a better marker

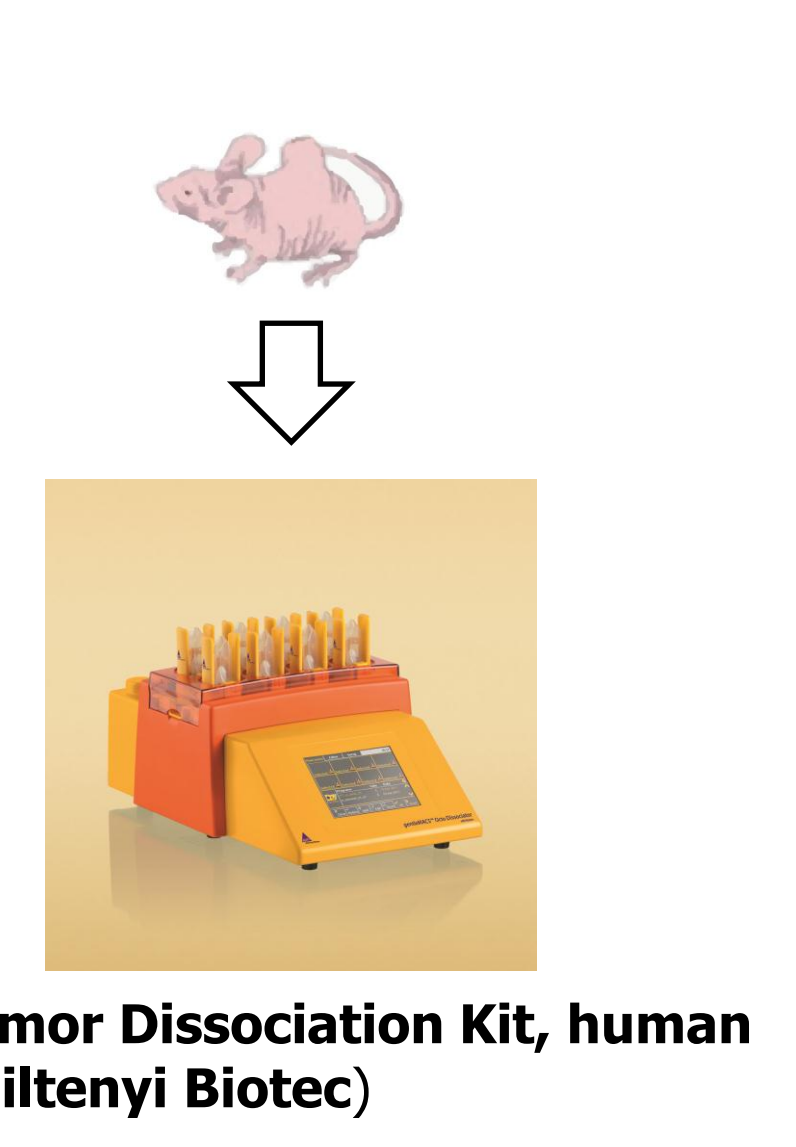
Marker ID (antibodies)	Gene symbol	Description	Marker ID (antibodies)	Gene symbol	Description
ABC5	ABC5	ATP-binding cassette, sub-family B of integral membrane proteins	CD34	CD34	highly glycosylated single-pass membrane protein
AN2/MCSP	CSPG4	an integral membrane chondroitin sulfate proteoglycan	CD340 (HER2/neu)	ERBB2	receptor tyrosine kinase EGF family
CaSR	CASR	calcium-sensing receptor	CD38	CD38	ectoenzyme
CD10	MME	a common acute lymphocytic leukemia antigen	CD44	CD44	cell-surface glycoprotein, receptor for hyaluronin acid
CD105 (Endoglin)	ENG	homodimeric transmembrane glycoprotein endoglin	CD49a	ITGA1	alpha 1 subunit of integrin receptors
CD117	KIT	type 3 transmembrane receptor for mast cell growth factor	CD49b	ITGA2	integrin, alpha 2 subunit
CD122	IL2RB	interleukin 2 receptor, beta	CD49c	ITGA3	integrin, alpha 3 subunit
CD133/1	PROM1	prominin 1, a pentagon transmembrane glycoprotein	CD49d	ITGA4	integrin, alpha 4 subunit of VLA-4 receptor
CD133/2	PROM1	prominin 1, a pentagon transmembrane glycoprotein	CD49e	ITGA5	integrin, alpha 5 subunit of fibronectin receptor
CD138	SDC1	syndecan, transmembrane (type 1) heparan sulfate proteoglycan	CD49f	ITGA6	integrin, alpha 6 subunit
CD146	MCAM	melanoma cell adhesion molecule	CD61	ITGB3	integrin, beta 3 subunit
CD15/SSEA1	FUT4	fucosyltransferase 4	CD66 (a,c,d,e)	CEACAM1	carcinoembryonic antigen-related cell adhesion molecule 1
CD166	ALCAM	activated leukocyte cell adhesion molecule	CD71	TFRC	transferrin receptor
CD20	MS4A1	membrane-spanning 4-domains, subfamily A, member 1	CD9	CD9	cell surface glycoprotein, tetraspanin protein family
CD24	CD24	cell surface sialoglycoprotein	CD90	THY1	Thy-1 cell surface antigen
CD26	DPP4	membrane glycoprotein	DRD5	DRD5	dopamine receptor D5
CD271	NGFR	nerve growth factor receptor	Lgr5 DA03	LGR5	leucine-rich repeat containing G protein-coupled receptor 5
CD309	KDR	kinase insert domain receptor tyrosine kinase	ROR1	ROR1	receptor tyrosine kinase-like orphan receptor 1
CD324-Ecad	CDH1	E-cadherin, a calcium dependent cell-cell adhesion glycoprotein	SSEA4	-	Sialylglycolectin/glyboside, stage-specific embryonic antigen 4
CD325-Ncad	CDH2	N-cadherin, a calcium dependent cell-cell adhesion glycoprotein	TGFbetaR	TGFBR1	a serine/threonine protein kinase
CD326	EPCAM	epithelial cell adhesion molecule	TRA-1-60	-	a cell surface antigen on undifferentiated human EC cells
CD338	ABC2	ATP-binding cassette, sub-family G (WHITE), member 2	TRA-1-81	-	Epitope associated with a keratin-sulfated transmembrane protein

### Study design for identifying new markers of chemotherapy-resistant cells

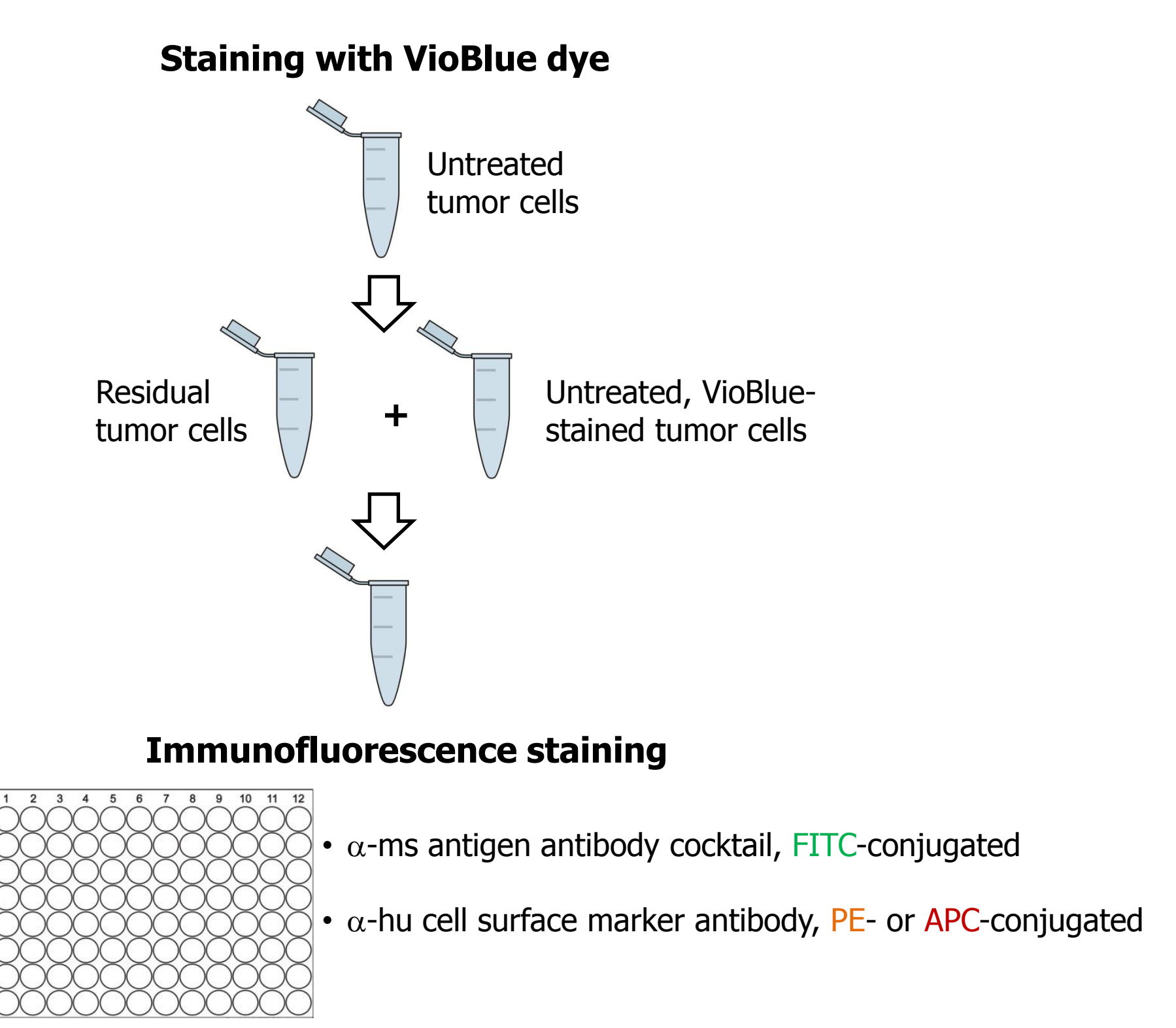
#### A. *In vivo* induction of PDX tumor regression and re-growth with adriamycin / cyclophosphamide chemotherapy



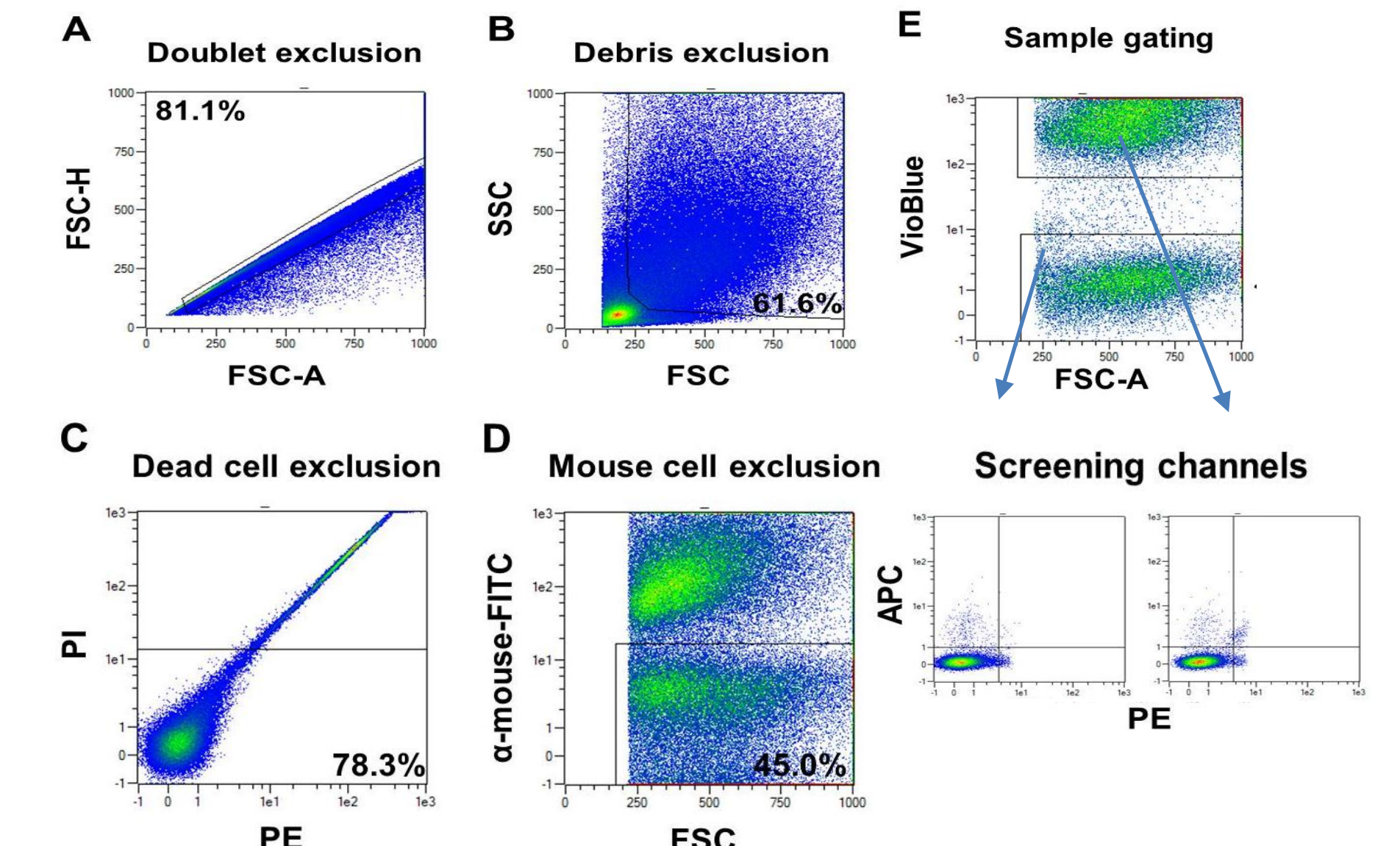
#### B. PDX tumor dissociation



#### C. Immunofluorescence staining

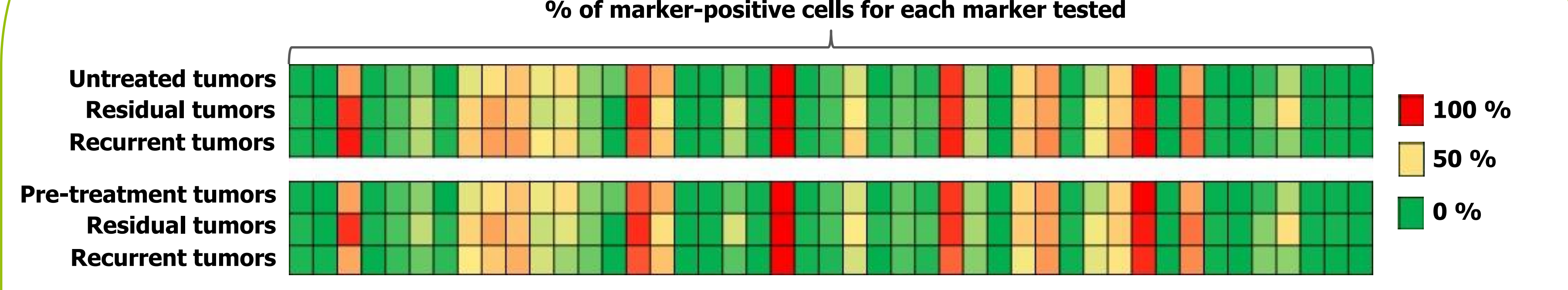


#### D. Gating strategy for flow cytometry-based cell surface marker expression analysis

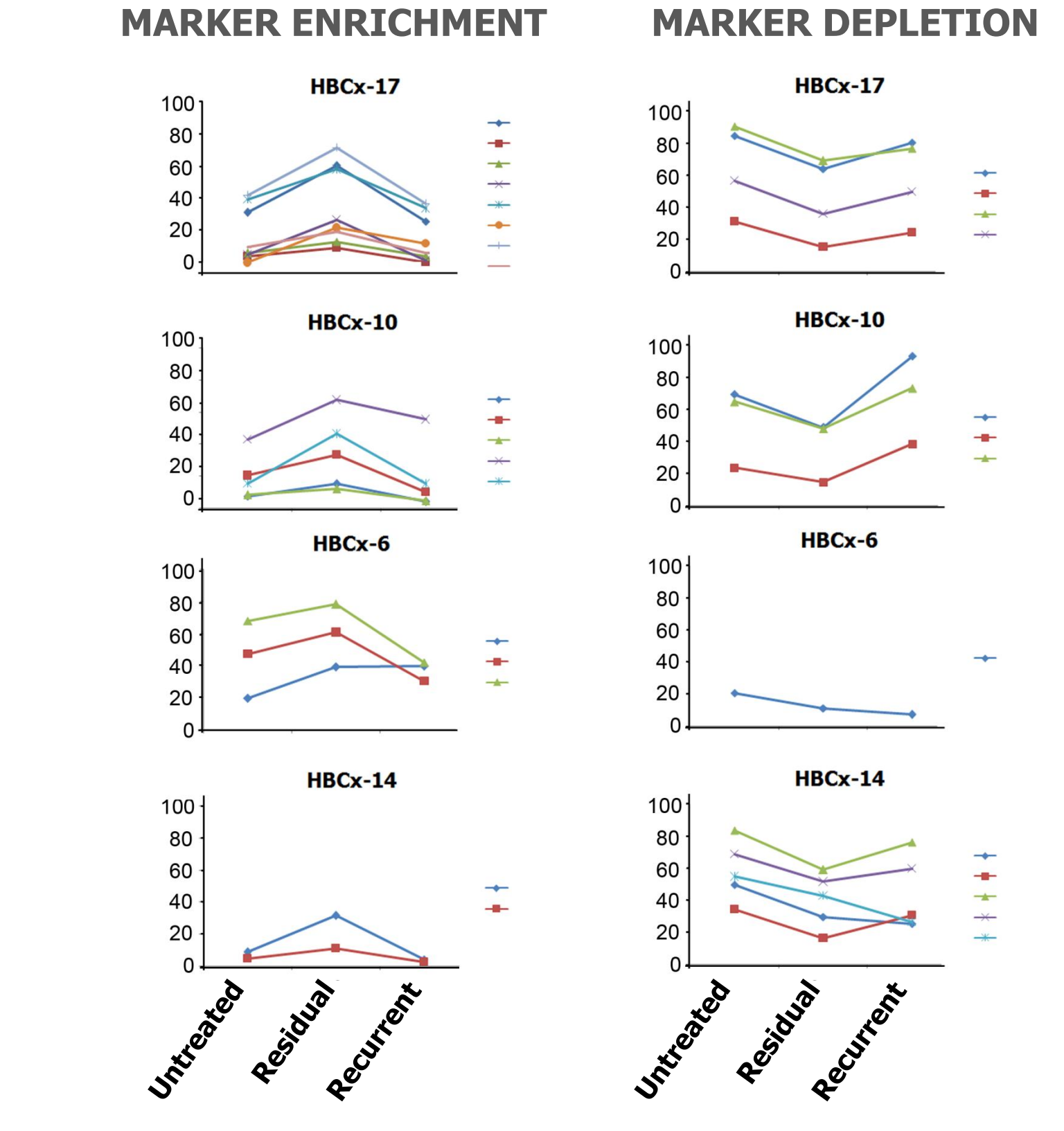


### Selection of candidate markers for molecular and functional characterization

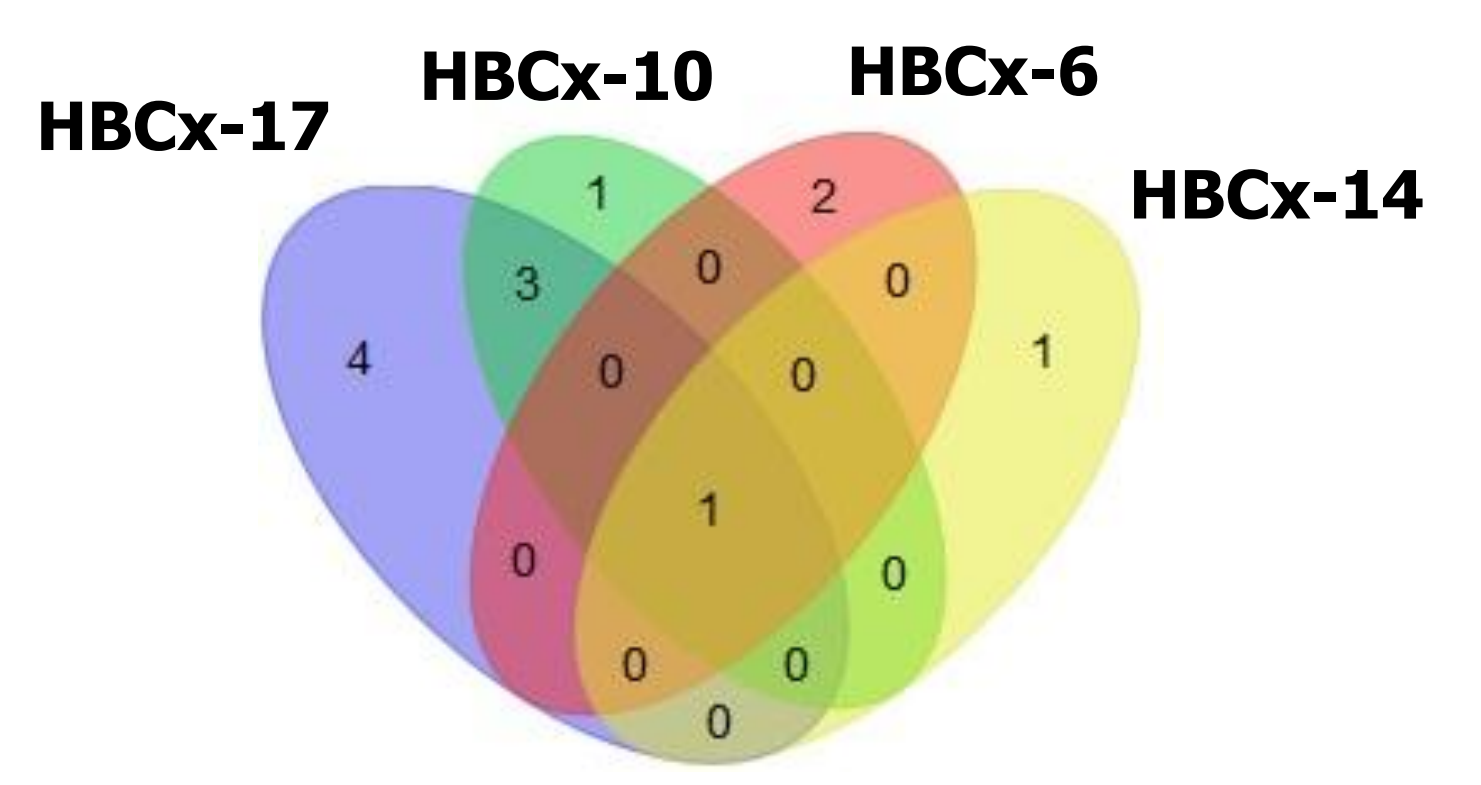
#### Cell surface marker expression before and after A/C chemotherapy: average of four different TNBC PDX models



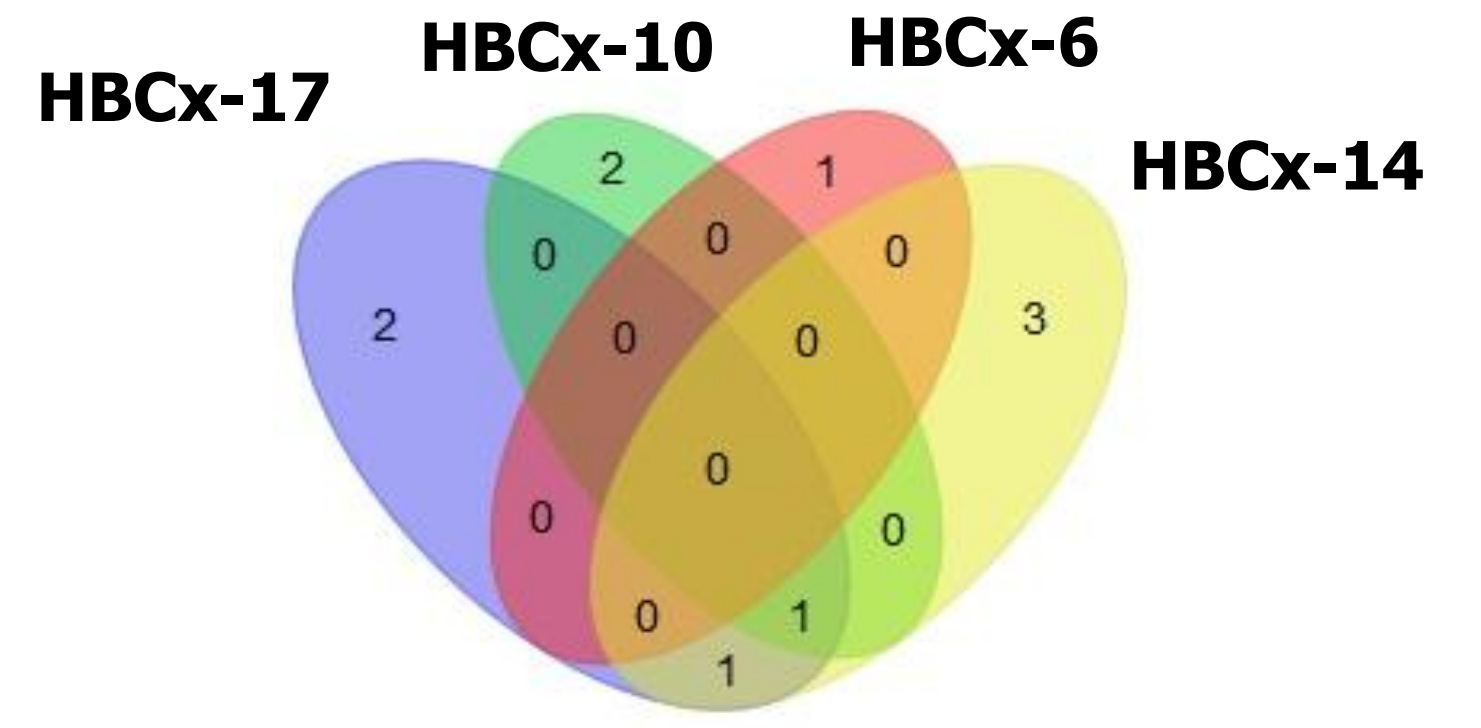
#### Candidate markers



#### One common marker enriched in residual tumor cells

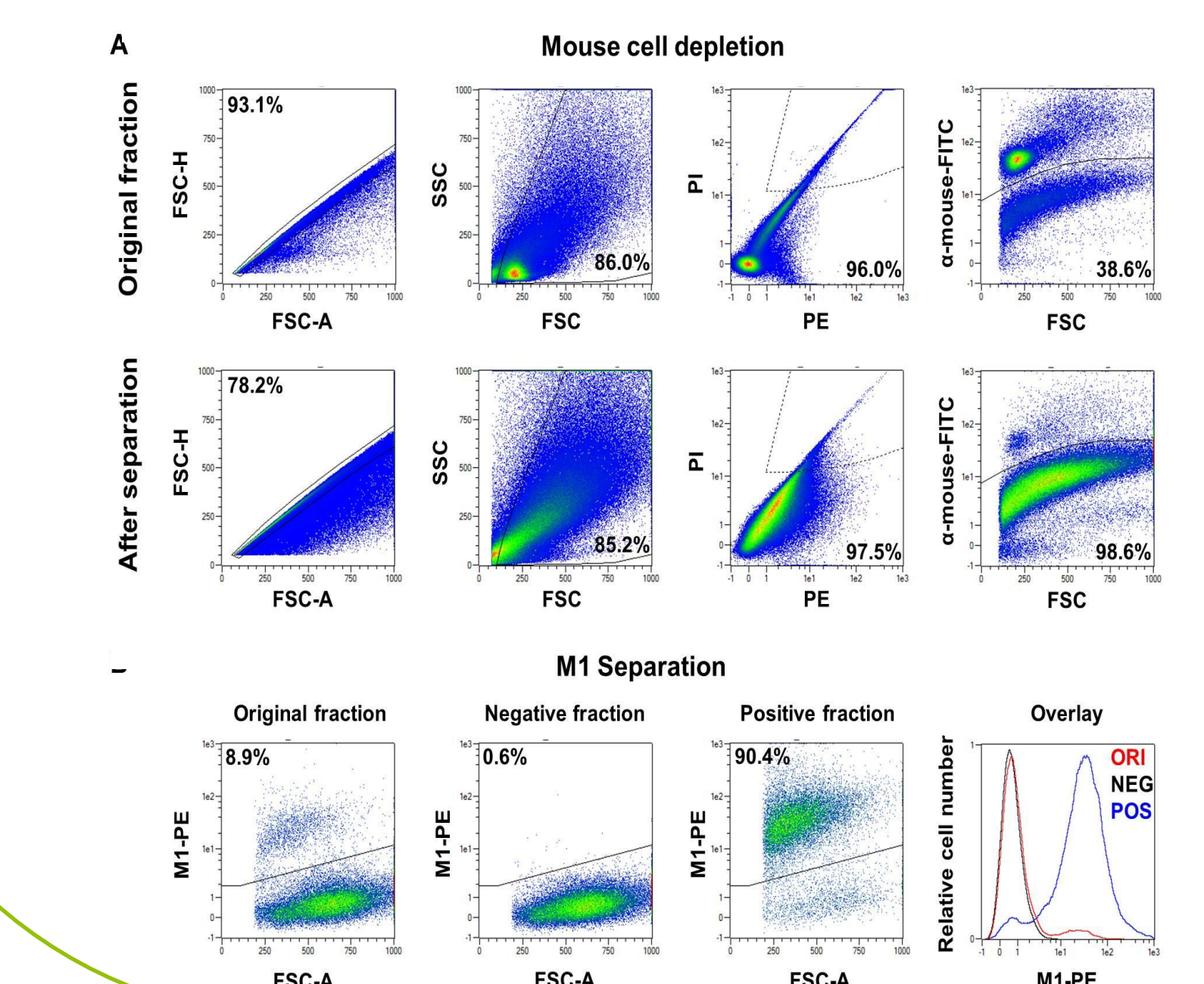


#### No common marker depleted in residual tumor cells

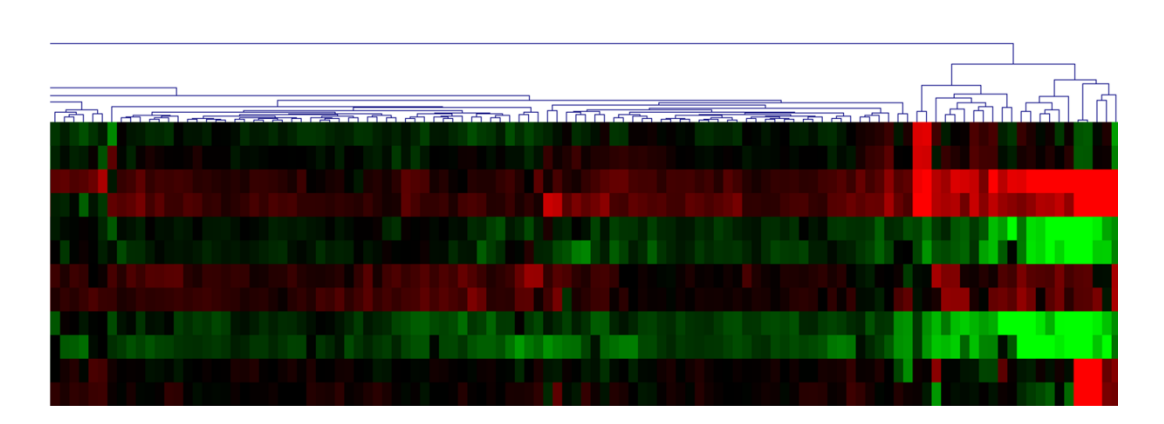


#### Molecular and functional analyses of sorted tumor cell sub-populations

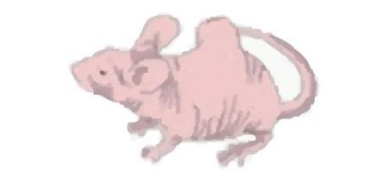
##### Strategy for flow cytometry analysis of PDX tumor cells before and after MACS-based sorting based on candidate marker expression



##### mRNA and miRNA expression analyses



##### *In vivo* tumor initiation and drug-response assays



##### *In vitro* sphere formation and drug cytotoxicity assays

