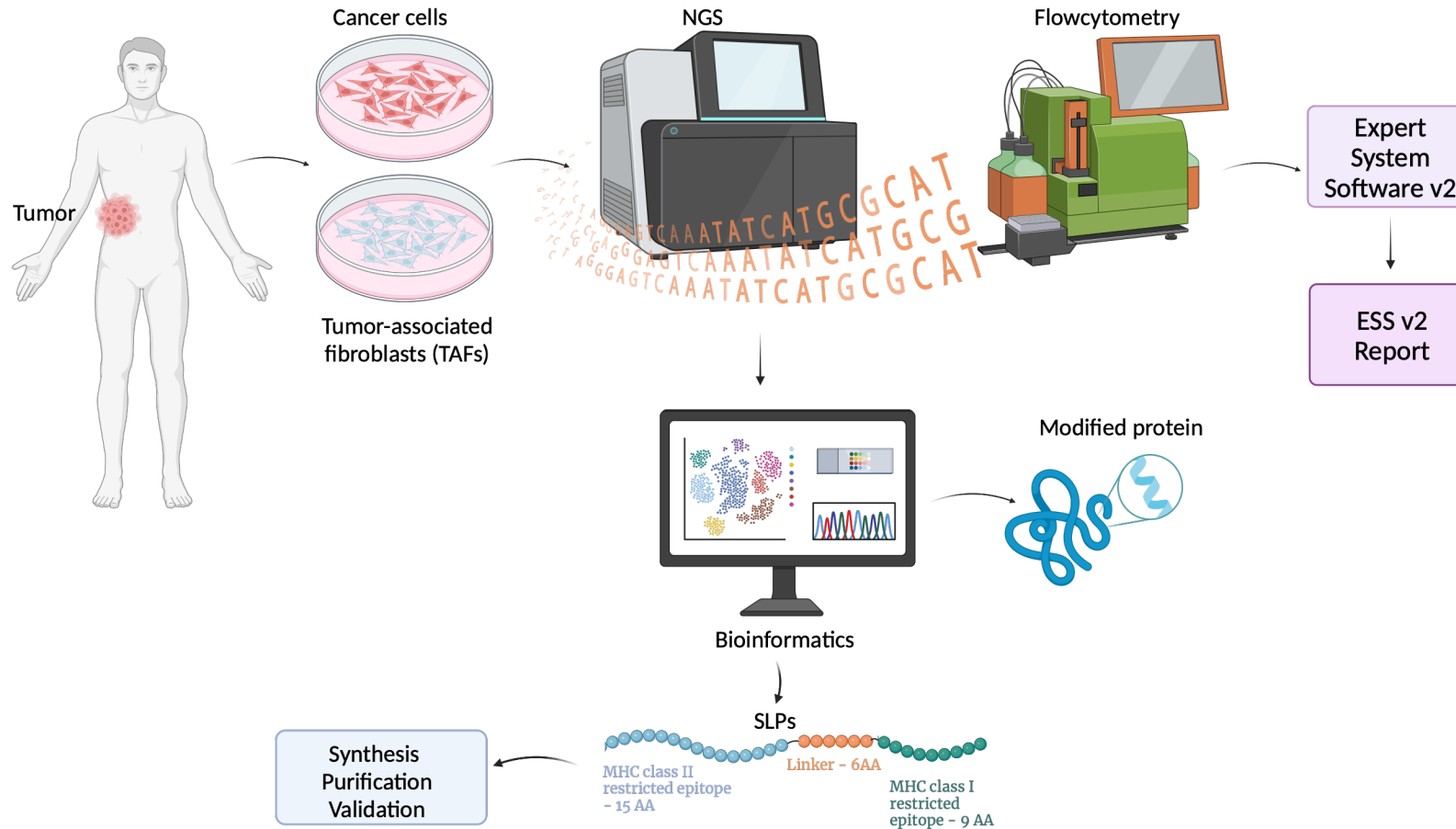


State of the art methods for tissue and cell processing for genomic, immunophenotypic and cytotoxicity assessment

Emergency Clinical County Hospital “Pius Brinzeu” Timisoara

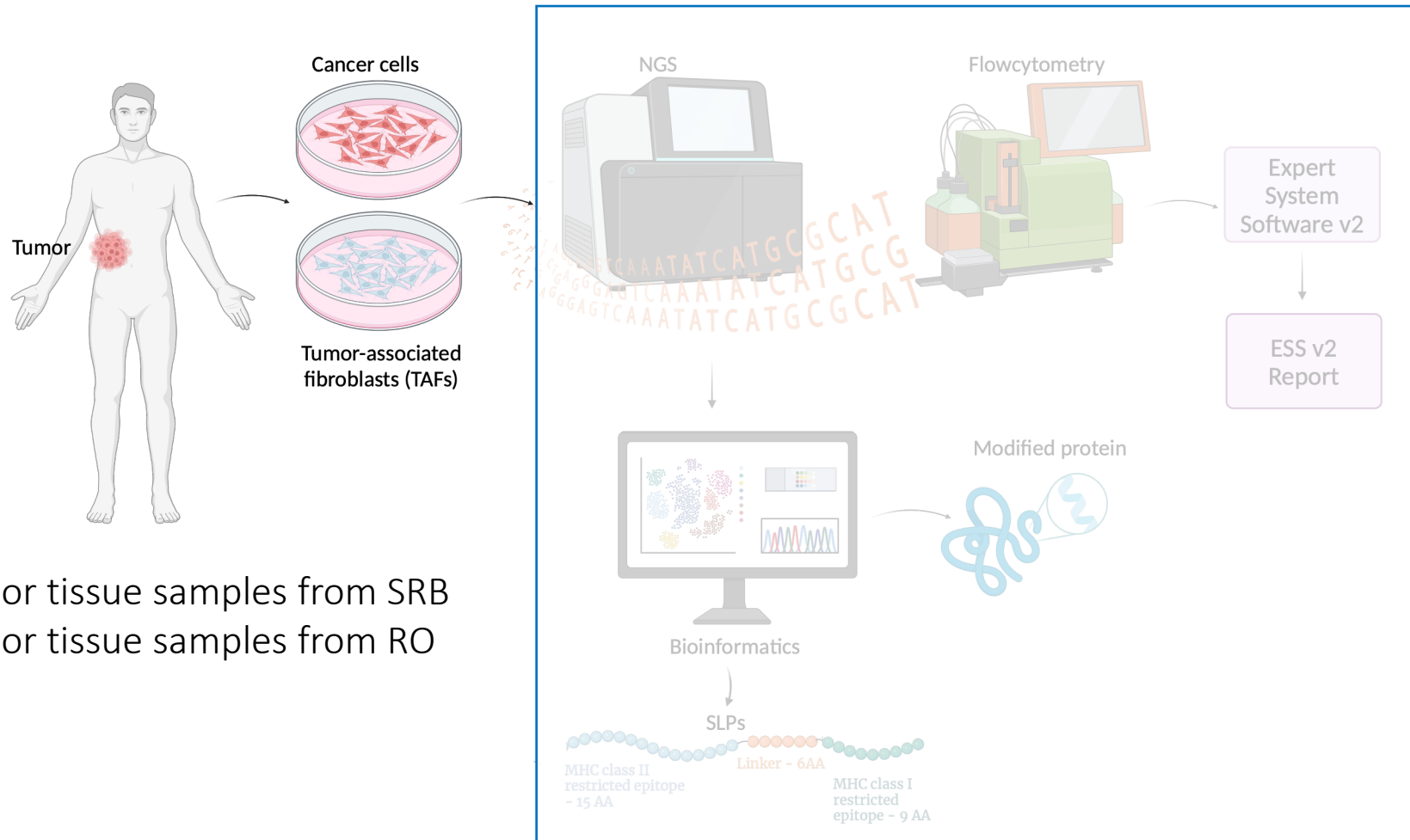
Medical and research team: Bojin Florina, Gavriliuc Oana, Cristea Mirabela, Buzan Roxana, Zbîrcea Lauriana, Grijincu Manuela, Simina Alina, Furdui Marieta, Șerban Daniela, Stanciu Claudia, Popoiu Tudor

The project



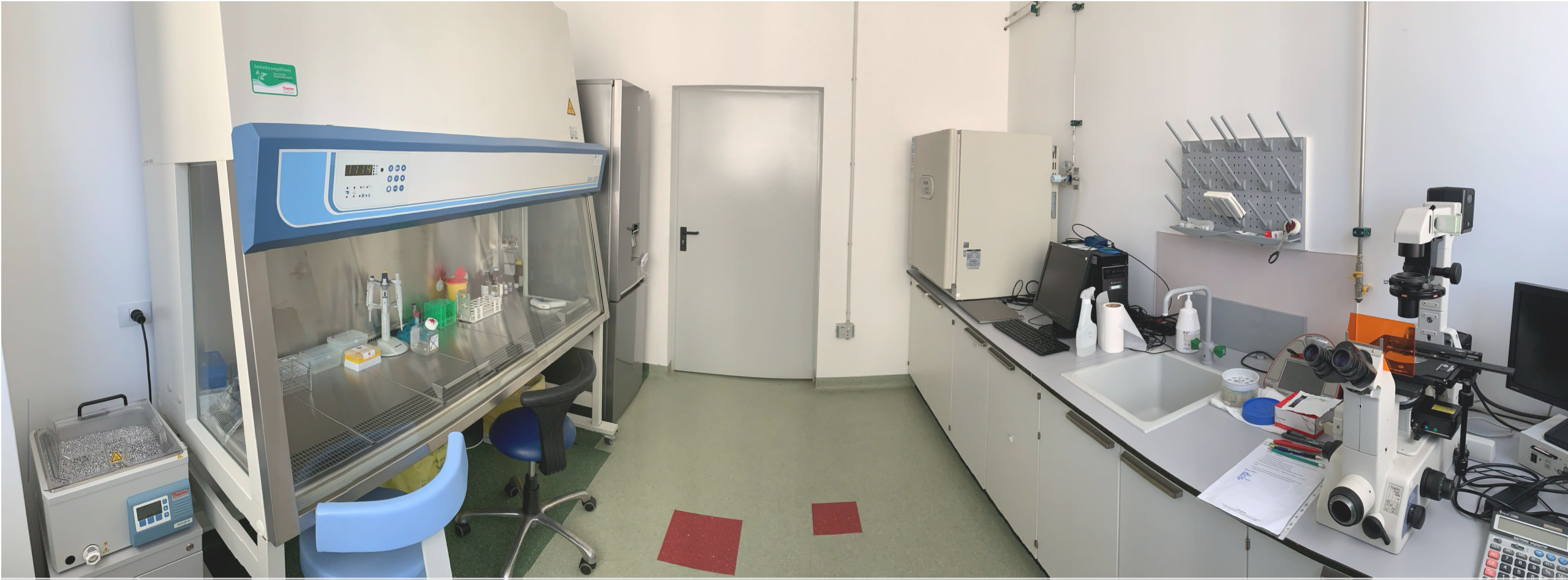
Timeline

Where we are

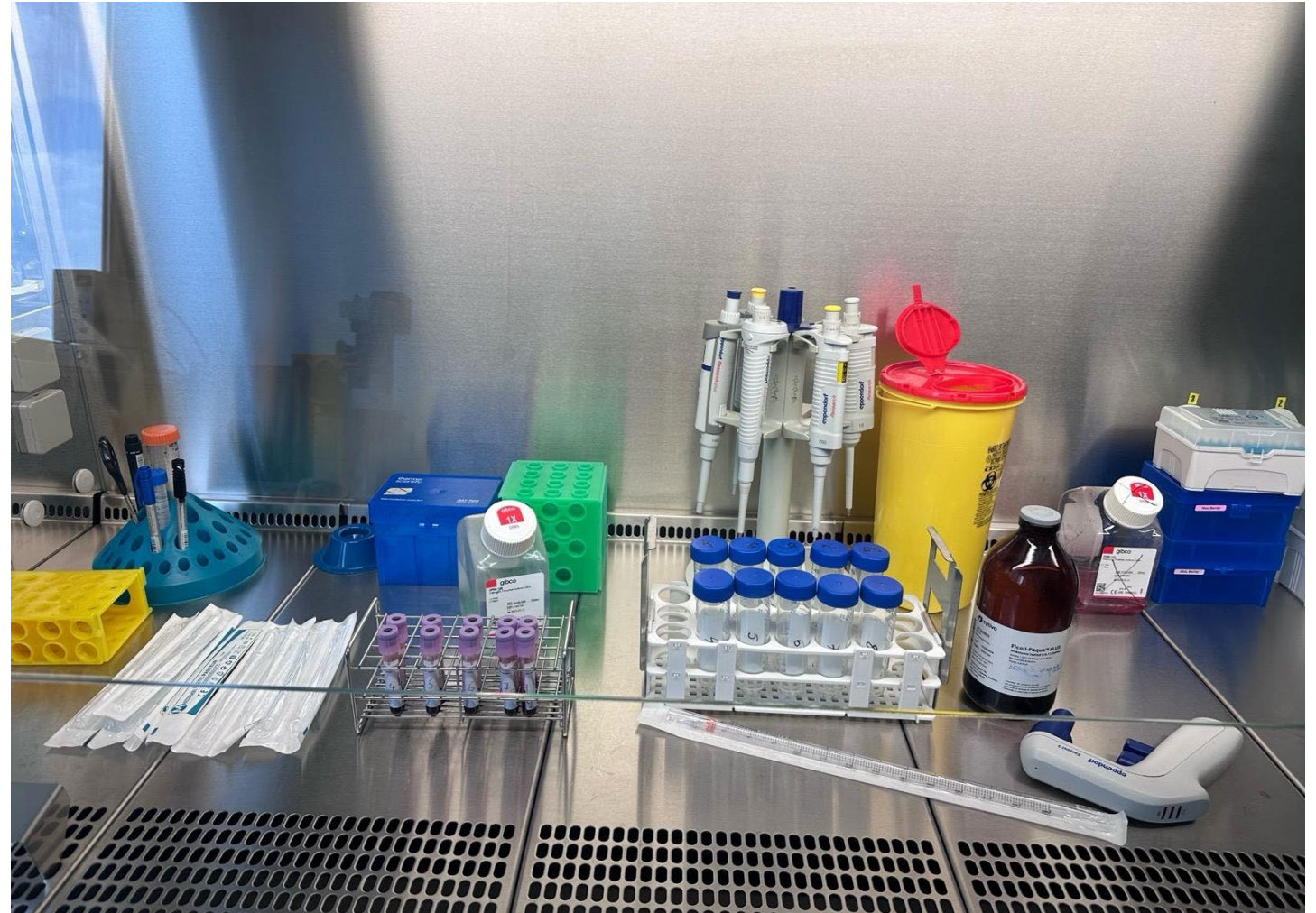


- 76 blood and tumor tissue samples from SRB
- 34 blood and tumor tissue samples from RO

Timeline

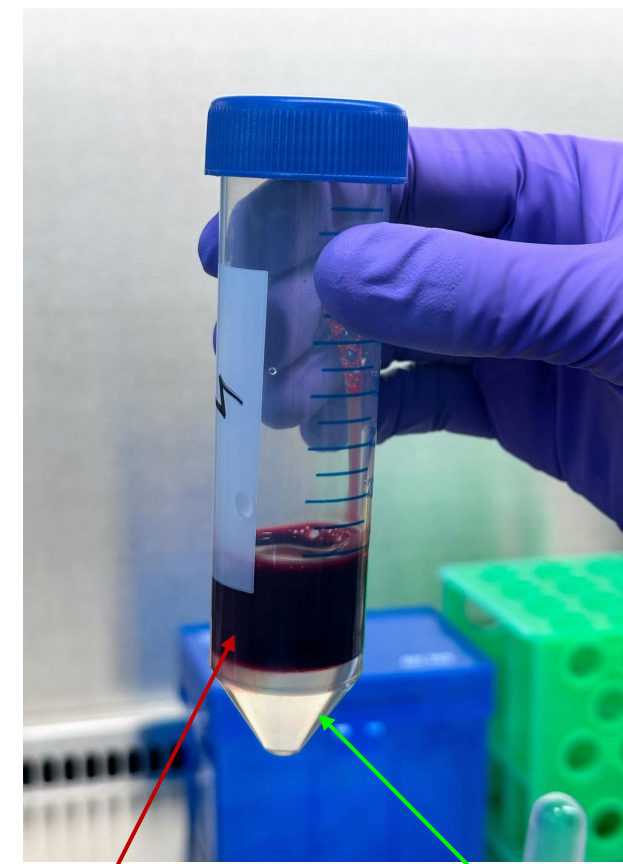


Blood samples processing



Blood samples processing

1. Dilute the blood sample 1 : 1 with sterile PBS at room temperature in a 50 ml Falcon tube and mix well by pipetting
2. Place in a 50 ml Falcon tube Ficoll (at room temperature) in a volume equal to the volume of the undiluted blood sample
3. Pipette the diluted blood sample into the Ficoll tube, carefully, on top, by tilting the Ficoll tube at an angle of approximately 45°, so that the entire amount of blood is above the Ficoll layer



Diluted blood

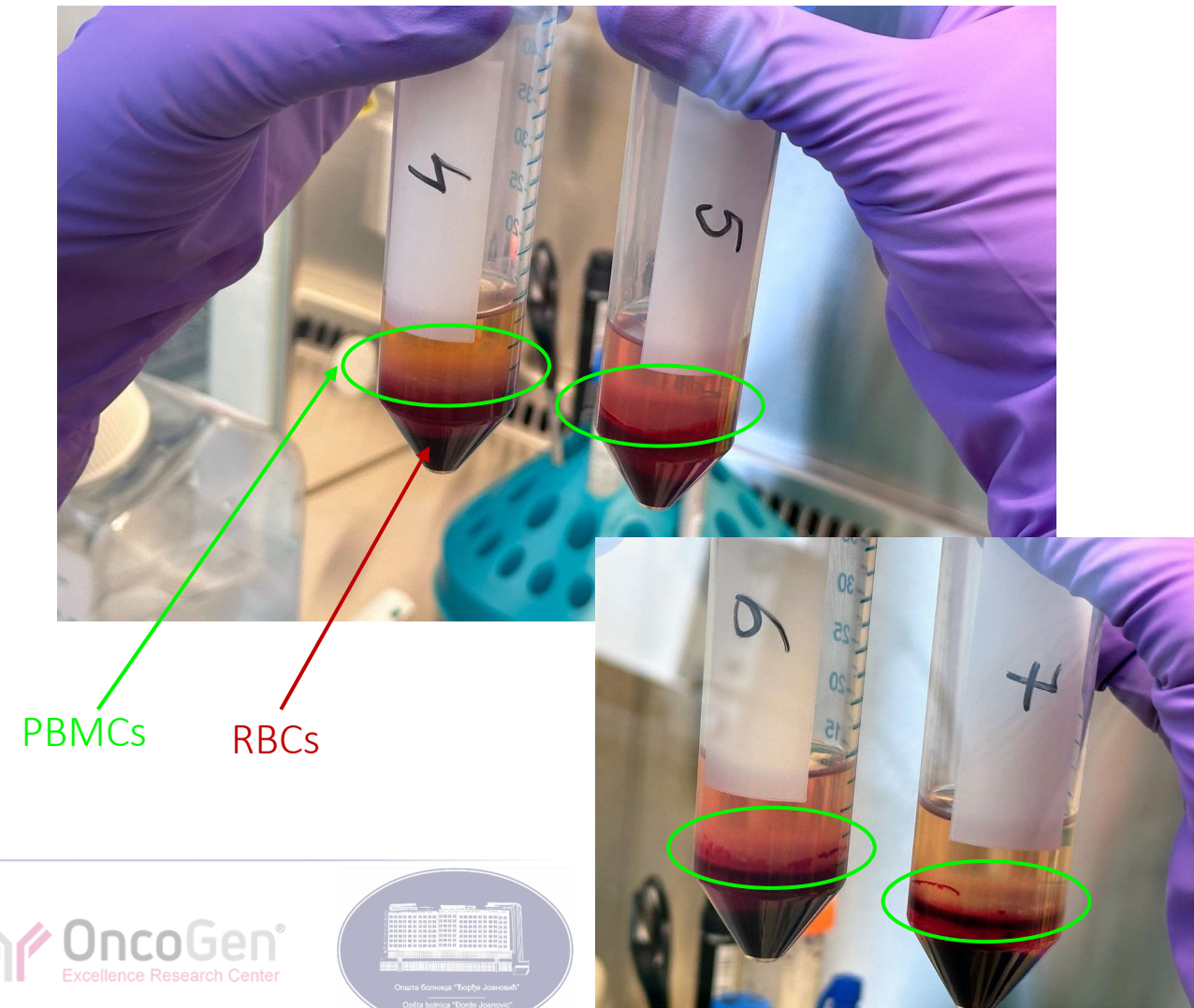
Ficoll

Blood samples processing



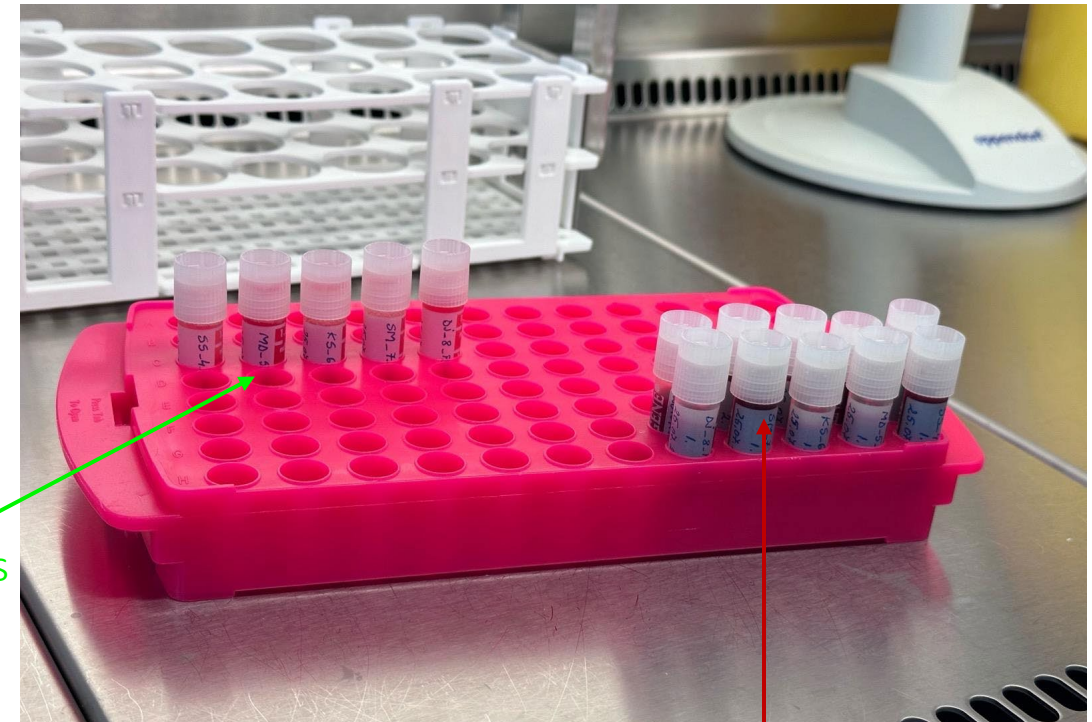
Blood samples processing

4. Centrifuge the Ficoll tube + diluted blood at 2500 rpm for 30 minutes without brake;
5. After centrifugation, the tube will be placed in the hood in a suitable support, in a vertical position, to visualize the layers of components: at the bottom – erythrocytes and platelets, granulocytes; Ficoll; mononuclear cell ring (PMBC); plasma



Blood samples processing

6. Collect the mononuclear cell ring from the Ficoll-plasma interface and transfer into another 50 ml Falcon tube;
7. Wash with PBS by centrifugation at 1500 rpm for 10 minutes
8. After washing, discard the supernatant and resuspend the sediment (pellet) in a volume of 1.8 ml in the following medium (warm, at 37° C): RPMI + 10% FCS + 1% Pen/Strep
9. Transfer the 1.8 ml volume to a cryogenic tube and add 200 µl DMSO (10% of the total volume); mix the contents of the tube quickly by rotating/inverting and place in the cryobox for long-term freezing, initially at -80° C, subsequently at -196° C (liquid nitrogen)



PBMCs

RBCs

Blood samples processing

Between steps 7 and 8 of the above protocol, **the erythrocytes** will be isolated and frozen as follows:

- Using a Pasteur pipette, the following layers remaining after harvesting the mononuclear cell ring will be removed: plasma and Ficoll; the bottom layer, composed predominantly of erythrocytes (RBCs), will remain
- From an initial blood volume of 2 ml, we will obtain 1 ml of RBC; from 4 ml of initial blood we will obtain approximately 2 ml of RBCs
- Add an equal volume of glycerol 40% over the volume of RBCs, mix well by pipetting and transfer to cryogenic tubes
- Insert the tubes into the cryobox and wait for the completion of the procedure for isolating PBMCs, to insert the cryobox into the deep freezer, at -80° C

Blood samples processing

Cryogenic tube labeling

- All cryogenic tubes will have the patient code, consisting of the initials of the name and the sample number from the Sample Reception register within the CrossCare project (e.g.: XY_15)
- Cryogenic tubes for PBMCs will be labeled as follows: patient code + PBMCs (e.g.: XY_15_PBMCs)
- Cryogenic tubes for RBCs will be labeled as follows: patient code + RBCs + tube no. (when there are 2 tubes of RBCs)(e.g.: XY_15_RBCs_1 or XY_15_RBCs_2)
- The date on which the products obtained from the venous blood samples were frozen will be noted on all tubes
- All this information (no. of tubes of each cell type, date of freezing, etc.) will also be noted in the CrossCare project register

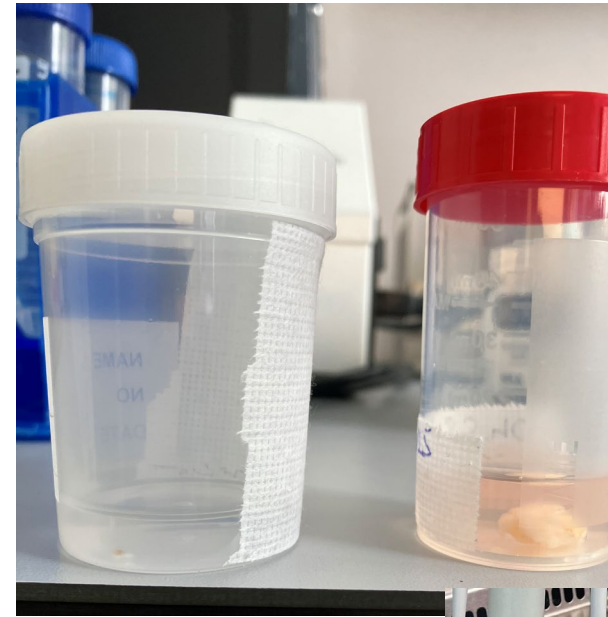
Labeling example:	XY_15_PBMCs	XY_15_RBCs_1	XY_15_RBCs_2
	25.07.2025	25.07.2025	25.07.2025

Tissue samples processing

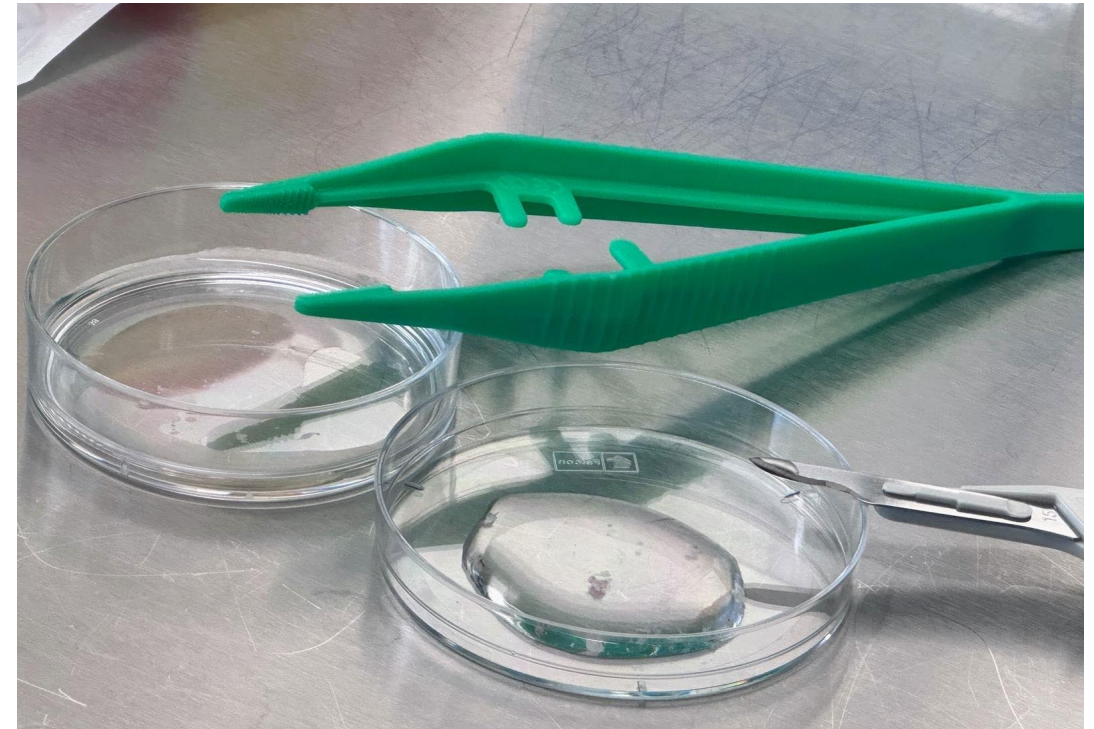


Tissue samples processing

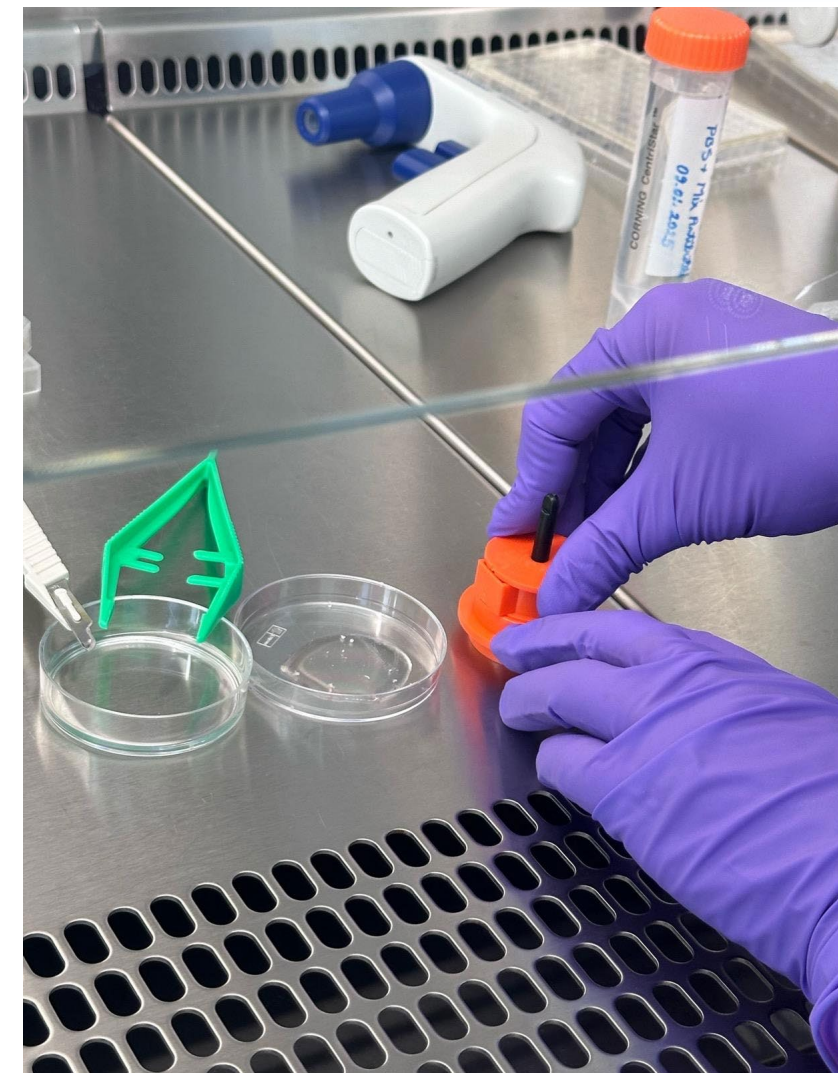
1. The fragments are of variable size and in variable number, transported in liquid medium (PBS + antibiotic and antifungal solution);
2. The fragments received are counted/measured and one fragment is selected which will be placed in a 1.5 ml tube with 500 μ l of RNA / DNA shield solution being used for DNA purification and subsequent use in the gene sequencing (NGS) protocol; The remaining fragments are divided in approximately equal parts for mechanical dissociation with the Medimachine device;
3. Carefully remove the biopsy fragments using a Pasteur pipette and place in a 3 cm / 5 cm ϕ Petri dish;



Tissue samples processing



Tissue samples processing



Tissue samples processing

4. Wash with PBS + antibiotic and antifungal solution for 15 minutes;
5. Remove half of the fragments and place in the sterile grinding chamber, adding 1 ml of PBS + antibiotic and antifungal solution (sterile); carefully close the grinding chamber and position in the Medimachine device;
6. Turn on the Medimachine device and leave the samples to grind for 3 minutes, then turn off the device; Carefully remove the grinding chamber and process the contents further in the laminar flow hood;



Tissue samples processing

7. Using a 1 ml syringe, remove the liquid fraction of the ground sample and filter it through filters compatible with 5 ml tubes, with a pore size of 50-100 μm ;
8. The larger, unground fragments are removed with a Pasteur pipette and placed in 24-well plate and left to dry;

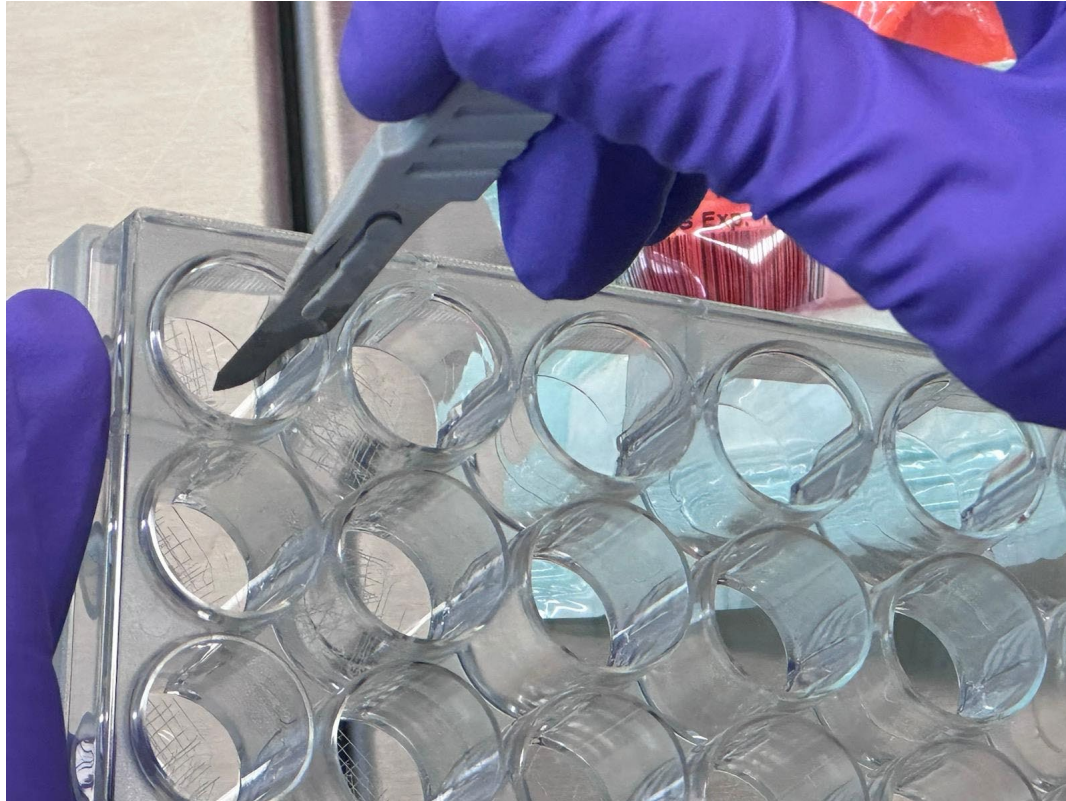


Tissue samples processing

9. Repeat steps 5 to 8 for the other part of the biopsy sample, using the same filter, but a different 5 ml tube; at the end of this step we will have 2 5 ml tubes containing 1 ml of cell suspension each and 4 wells with solid biopsy samples;
10. Add 1 ml of PBS + antibiotic/antifungal solution to each tube and centrifuge the 2 5 ml tubes with the cell suspension at 1500 rpm for 10 minutes at room temperature.

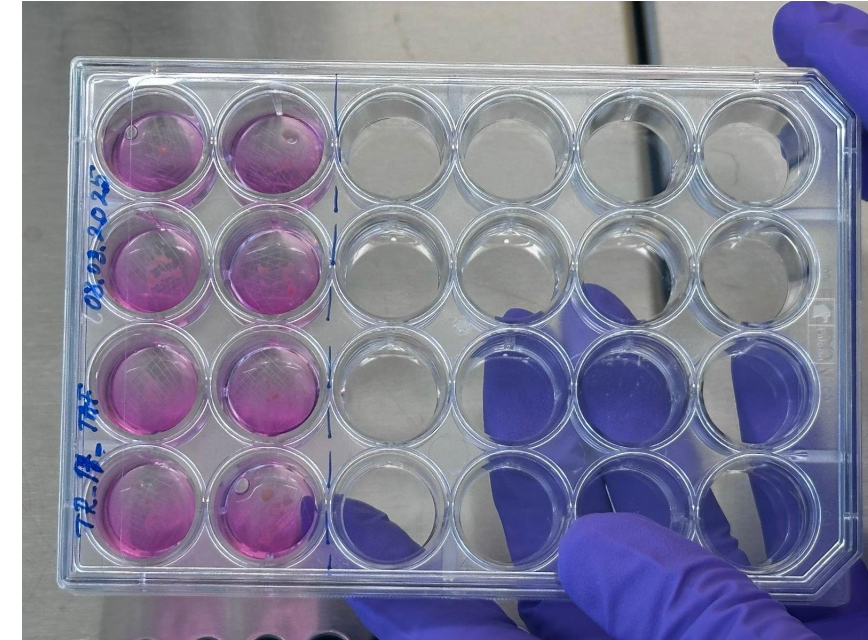


Tissue samples processing



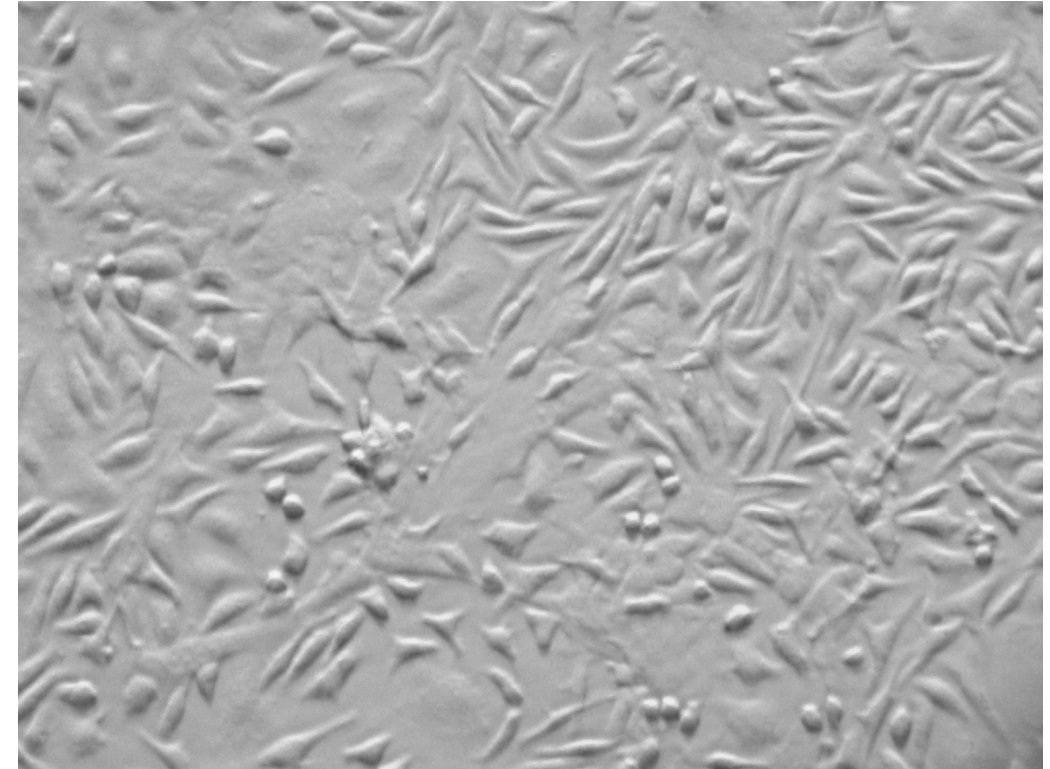
Tissue samples processing

11. Carefully add (without removing the solid fragments previously placed in wells) 1 ml of TAFs medium/well: DMEM + 10% FCS + 1% Pen/Strep + 10 ng/ml FGF



Tissue samples processing

11. At the end of the procedure, we will have 4 wells occupied in the 24-well plate with peritumoral fibroblasts (TAFs);
12. The culture plate is positioned in the incubator, at 37°C with 5% CO₂ and humidity;
13. The culture plate medium is checked daily to monitor the evolution of the cells and to detect the appearance of infections early (especially in the case of colon tumors); The medium is changed after 7 days from seeding; solid fragments are removed from the culture plate after 4-10 days and the cultivation of is continued until the cells are reaching confluence.



Tissue samples processing

Labeling of culture plates/cryogenic tubes

- All plates (wells) and subsequently cryogenic tubes will have the patient code, formed by the initials of the name and the sample number from the Sample Reception register within the CrossCare project (e.g.: XY_15)
- The cryogenic tubes for TCs will be labeled as follows: patient code + TCs + tube no. (e.g.: XY_15_TC_1 and XY_15_TC_2 etc.)
- The cryogenic tubes for TAFs will be labeled as follows: patient code + TAFs + no. tube (e.g.: XY_15_TAFs_1 and XY_15_TAFs_2 etc.)
- All tubes will be marked with the date on which the products obtained from fresh tumor tissue samples were frozen;
- All this information (no. of tubes of each cell type, date of freezing etc.) will also be noted in the CrossCare project register

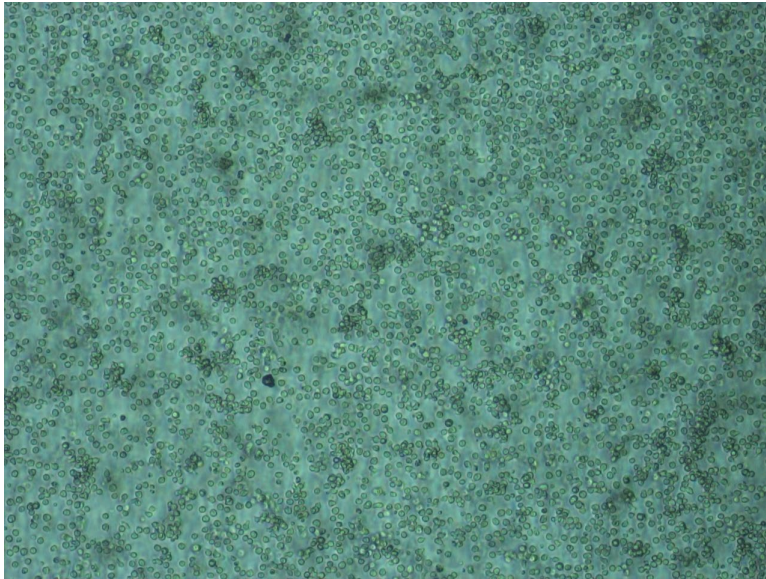
Labeling example:

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25.07.2025

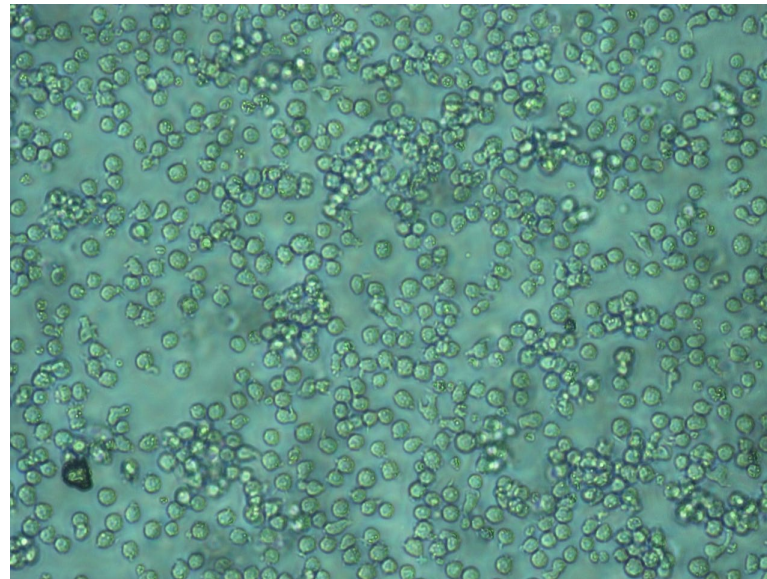
XY_15_TC_2
25.07.2025

XY_15_TAFs_1
10.08.2025

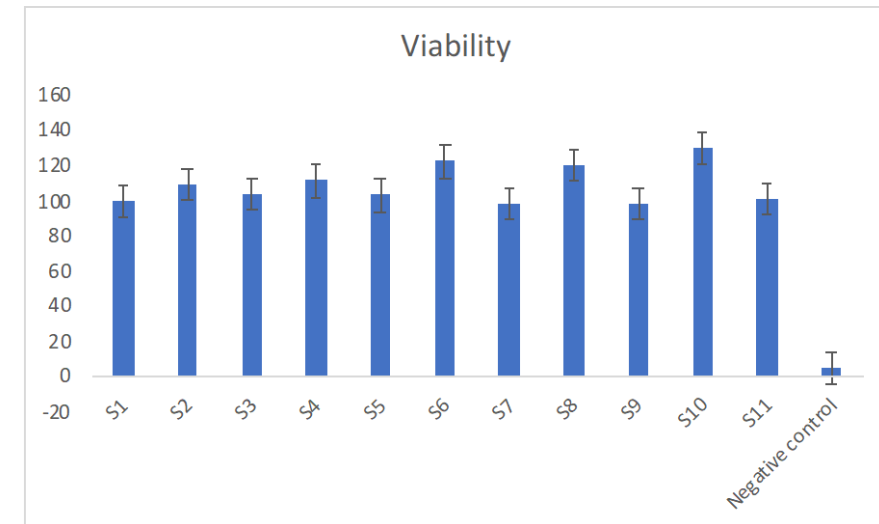
Cell analysis - viability



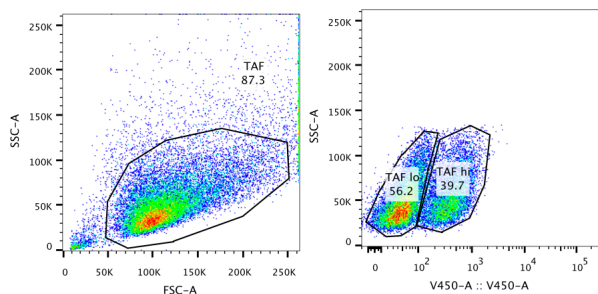
PBMCs, Ob. 4x



PBMCs, Ob. 10x

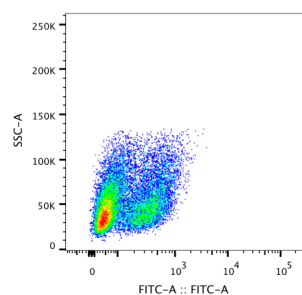


Cell analysis - flowcytometry

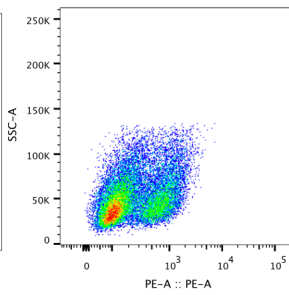


NM.fcs
Ungated
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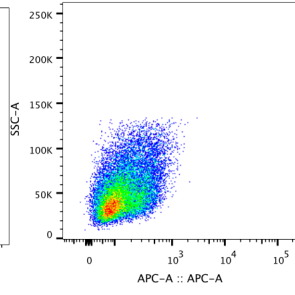
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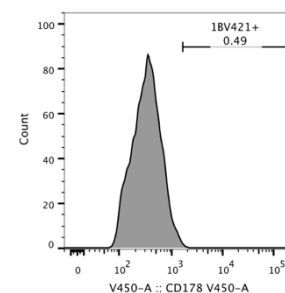
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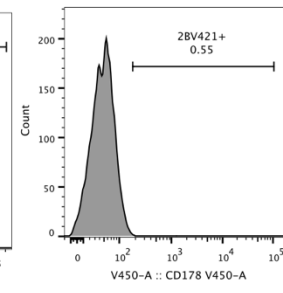
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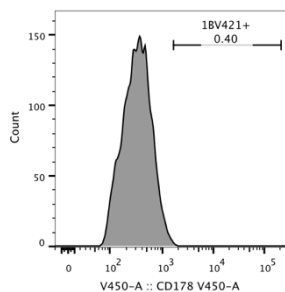
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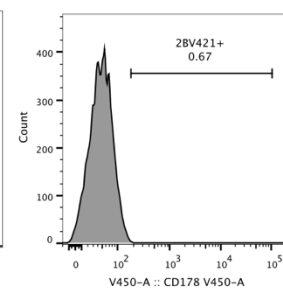
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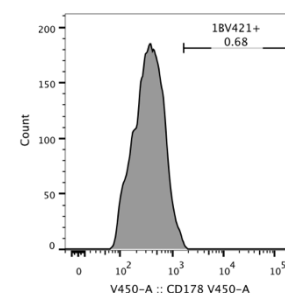
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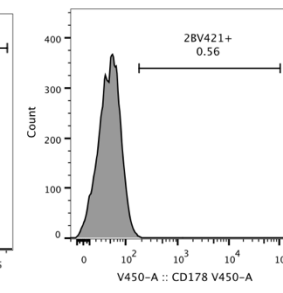
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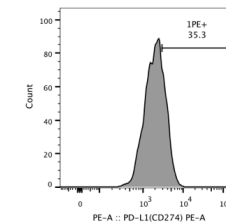
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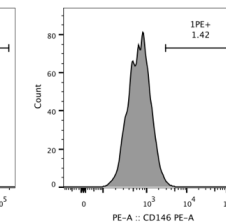
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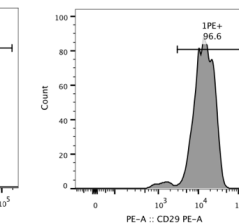
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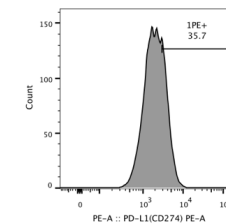
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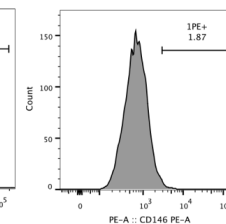
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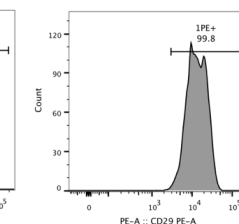
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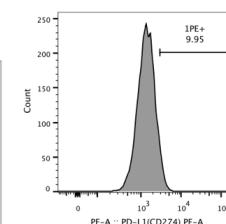
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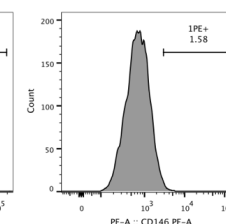
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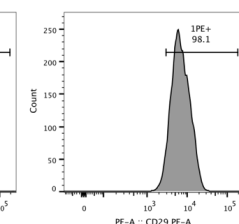
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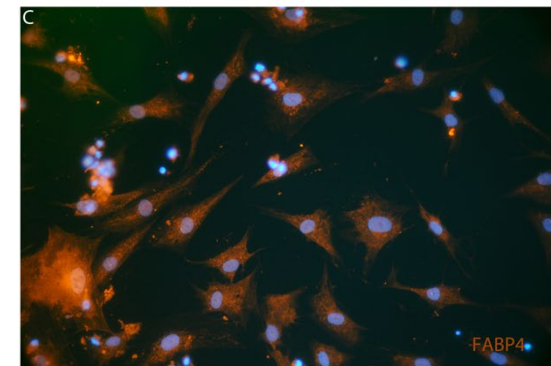
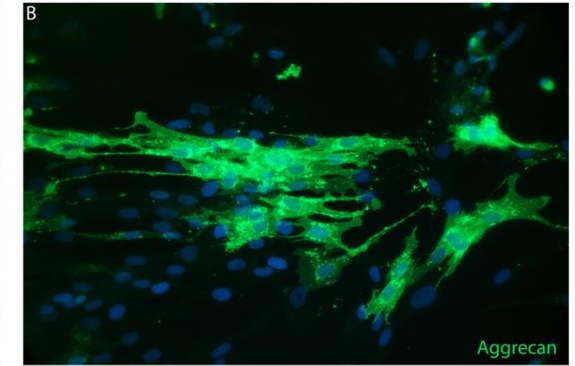
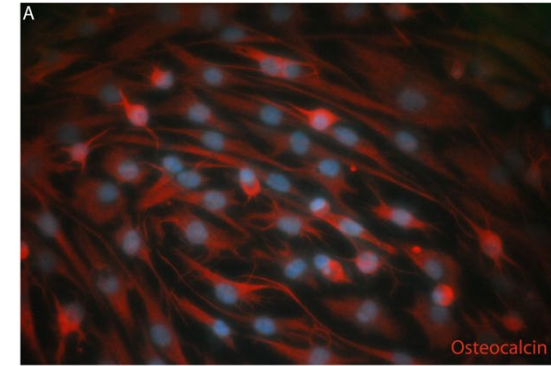
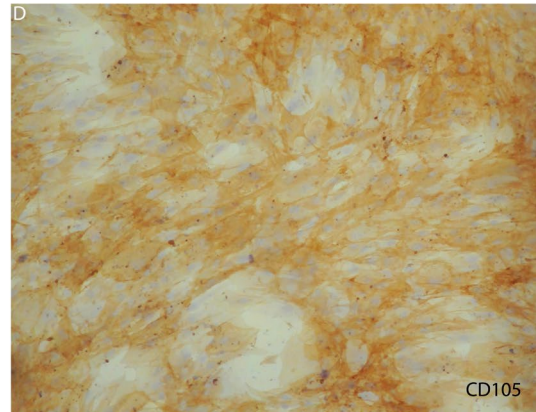
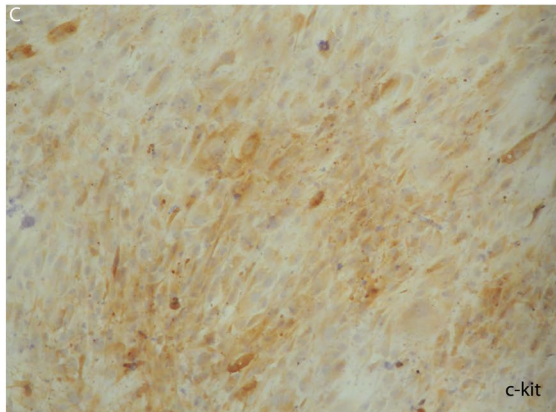
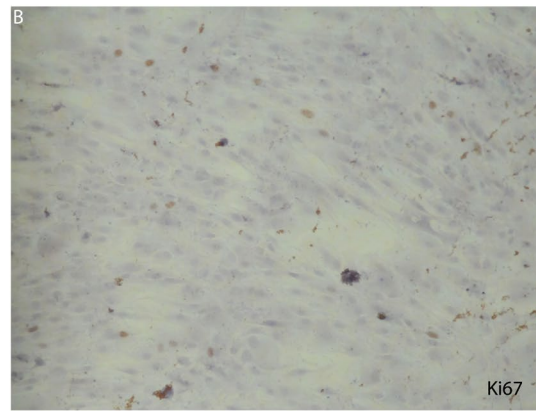
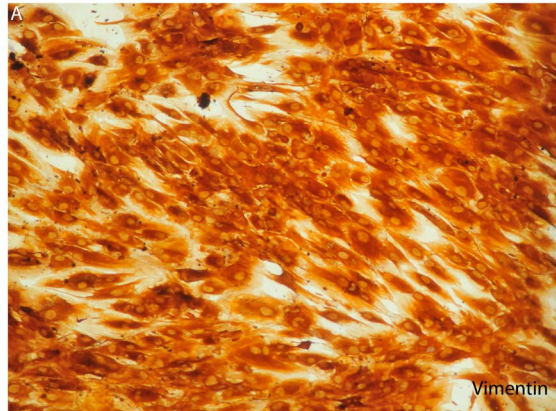


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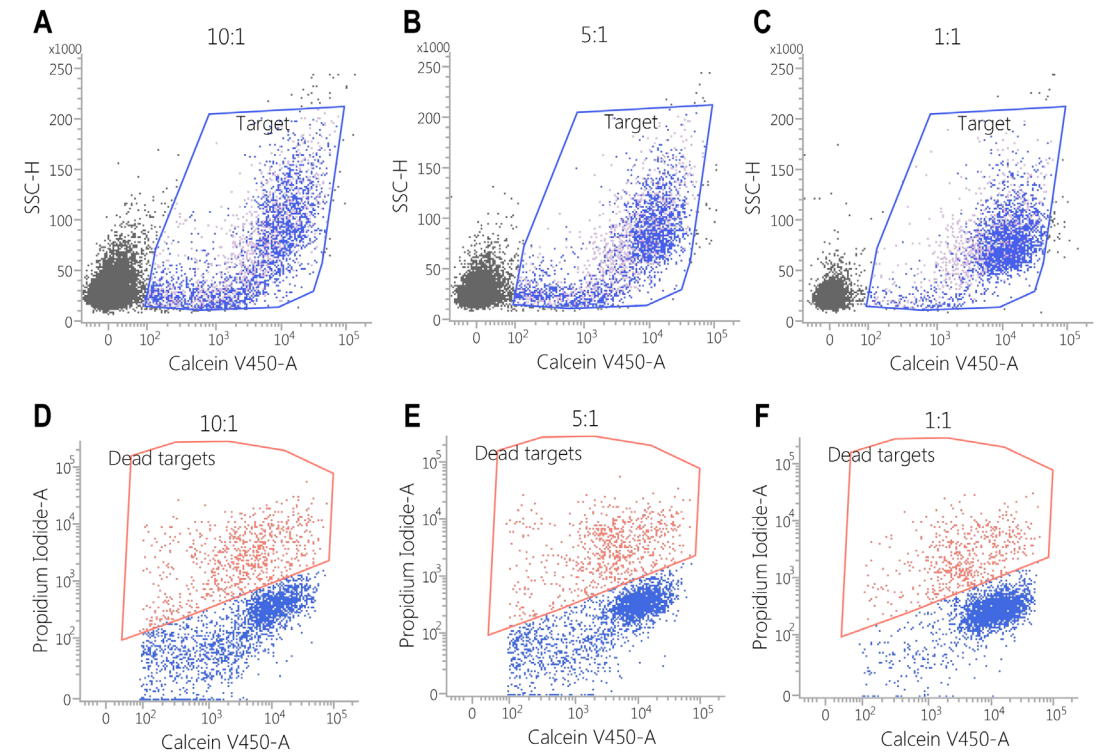
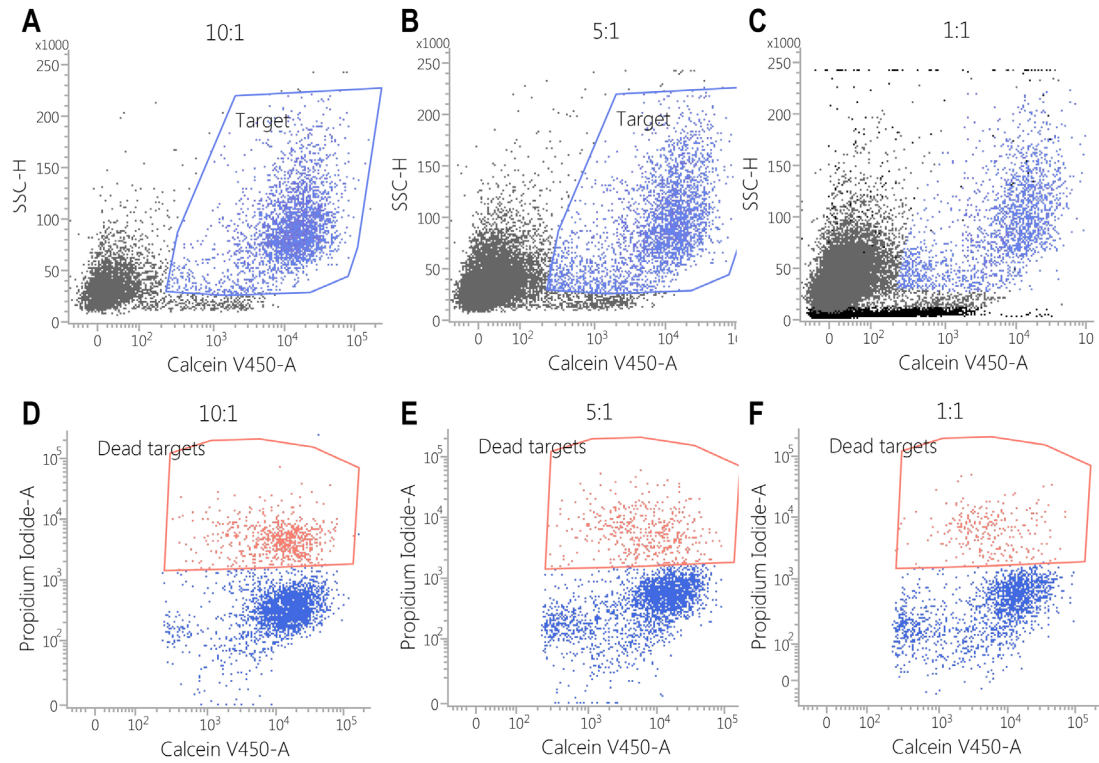


DR_CD29.fcs
TAF hi
8216

Cell analysis – immunocytochemistry and immunofluorescence



Cell analysis – cytotoxicity assay



Cell analysis – Next generation Sequencing (NGS)

chr3:41261:SNV	G	1	G/A	PASS	0.00249	566	G=553,A=1	G=0.977,A=	2.3	CTNNB1	NM_00190	CTNNB1:exonic:NM_001904.3	missense	GAT	3	p.Gly38Asp	c.113G>A
chr3:41261:SNV	C	1	C/T	PASS	3.49E-05	564	C=547,T=1	C=0.9699,T=	3.01	CTNNB1	NM_00190	CTNNB1:exonic:NM_001904.3	missense	GTT	3	p.Ala43Val	c.128C>T
chr3:17891:SNV	A	1	A/G	PASS	0	1036	A=460,G=5	A=0.444,G=	55.6	PIK3CA	NM_00621	PIK3CA:exonic:NM_006218.3	missense	ATG	7	p.Ile391Met	c.1173A>G
chr3:17891:SNV	C	1	C/T	PASS	0.072637	976	C=962,A=0	C=0.9857,A=	0.00	PIK3CA	NM_00621	PIK3CA:exonic:NM_006218.3	missense	ATC	21	p.Thr1025Ile	c.3074C>T
chr4:1806:SNV	T	1	T/C	PASS	0	700	T=154,C=5	T=0.22,C=0	78	FGFR3	NM_00014	FGFR3:exonic:NM_000142.4	missense	CTC	9	p.Phe384Leu	c.1150T>C
chr4:5559:SNV	A	1	A/C	PASS	0	1685	A=359,C=1	A=0.2131,C=	78.69	KIT	NM_00022	KIT:exonic:NM_000222.2	missense	CTG	10	p.Met541Leu	c.1621A>C
chr4:5594:SNV	C	1	C/T	PASS	6.24E-05	448	C=426,T=2	C=0.9509,T=	4.91	KDR	NM_00225	KDR:exonic:NM_002253.2	missense	AGA	30	p.Gly1333Arg	c.3997G>A
chr4:5595:SNV	C	1	C/T	PASS	0.156362	1375	C=1358,T=	C=0.9876,T=	1.24	KDR	NM_00225	KDR:exonic:NM_002253.2	missense	GAG	26	p.Gly1145Glu	c.3434G>A
chr4:5597:SNV	T	1	T/A	PASS	0	1105	T=274,A=8	T=0.248,A=	75.2	KDR	NM_00225	KDR:exonic:NM_002253.2	missense	CAT	11	p.Gln472His	c.1416A>T
chr4:5598:SNV	C	1	C/T	PASS	0.002389	881	C=851,T=3	C=0.9659,T=	3.41	KDR	NM_00225	KDR:exonic:NM_002253.2	missense	AAA	6	p.Glu261Lys	c.781G>A
chr4:1532:SNV	G	1	G/A	PASS	0.078184	745	G=734,A=1	G=0.9852,A=	1.48	FBXW7	NM_03363	FBXW7:exonic:NM_033632.3	missense	TGT	9	p.Arg465Cys	c.1393C>T
chr5:1121:SNV	C	1	C/T	PASS	1.29E-04	1121	C=1080,T=	C=0.9634,T=	3.66	APC	NM_00003	APC:exonic:NM_000038.5	missense	CTA	16	p.Pro1441Leu	c.4322C>T
chr5:1121:SNV	G	1	G/A	PASS	3.22E-04	1134	G=1094,A=	G=0.9647,A=	3.53	APC	NM_00003	APC:exonic:NM_000038.5	missense	ACT	16	p.Ala1460Thr	c.4378G>A
chr5:1121:SNV	G	1	G/A	PASS	0.007156	1825	G=1796,A=	G=0.9841,A=	1.59	APC	NM_00003	APC:exonic:NM_000038.5	missense	AGA	16	p.Gly1499Arg	c.4495G>A
chr7:5521:SNV	G	1	G/A	PASS	0.001484	2000	G=1966,A=	G=0.983,A=	1.7	EGFR	NM_00522	EGFR:exonic:NM_005228.4	missense	AAA	3	p.Arg108Lys	c.323G>A
chr7:5524:SNV	CATCTCCG	1	CATCTCCG	PASS	0.104752	1719	CATCTCCG	CATCTCCG	AAAGCAAC	EGFR	NM_00522	EGFR:exonic:NM_005228.4	missense	ATA	19	p.Thr751Ile	c.2252C>T
chr7:5524:SNV	G	1	G/A	PASS	0.072983	290	G=281,A=9	G=0.969,A=	3.1	EGFR	NM_00522	EGFR:exonic:NM_005228.4	missense	ATA	20	p.Met761Ile	c.2298G>A
chr7:5524:SNV	G	1	G/A	PASS	0.031313	287	G=281,A=6	G=0.9791,A=	2.09	EGFR	NM_00522	EGFR:exonic:NM_005228.4	missense	TAC	20	p.Cys775Tyr	c.2324G>A
chr9:2197:SNV	G	1	G/A	PASS	0.11221	1999	G=1974,A=	G=0.9875,A=	1.25	CDKN2A	NM_00119	CDKN2A:exonic:NM_001195132.1	missense	GTC	2	p.Ala76Val	c.227C>T
chr10:896:SNV	GGTTTTTG	1	GGTTTTTG	PASS	5.68E-06	1742	GGTTTTTG	GGTTTTTG	AGTTTGGATCAAGC	PTEN	NM_00031	PTEN:exonic:NM_000314.6	missense	TAT	3	p.His61Tyr	c.181C>T
chr10:896:SNV	G	1	G/A	PASS	0.004561	1537	G=1511,A=	G=0.9831,A=	1.69	PTEN	NM_00031	PTEN:exonic:NM_000314.6	missense	TAT	5	p.Cys105Tyr	c.314G>A
chr11:108:SNV	G	1	G/A	PASS	1.86E-04	1897	G=1836,A=	G=0.9678,A=	3.22	ATM	NM_00005	ATM:exonic:NM_000051.3	missense	AGA	34	p.Gly1676Arg	c.5026G>A
chr11:108:SNV	C	1	C/T	PASS	0.109515	1470	C=1451,T=	C=0.9871,T=	1.29	ATM	NM_00005	ATM:exonic:NM_000051.3	missense	TGT	63	p.Arg3008Cys	c.9022C>T
chr12:112:SNV	G	1	G/A	PASS	3.50E-06	788	G=765,A=2	G=0.9708,A=	2.92	PTPN11	NM_00283	PTPN11:exonic:NM_002834.4	missense	GAG	13	p.Gly503Glu	c.1508G>A
chr14:105:SNV	C	1	C/T	PASS	0.005317	1038	C=1019,T=	C=0.9817,T=	1.83	AKT1	NM_00101	AKT1:exonic:NM_001014431.1	missense	AAG	3	p.Glu49Lys	c.145G>A
chr17:757:SNV	C	1	C/T	PASS	0.138578	1002	C=989,T=1	C=0.987,T=	1.3	TP53	NM_00054	TP53:exonic:NM_000546.5	missense	GAG	8	p.Gly302Glu	c.905G>A
chr17:757:SNV	GGAGATTC	1	GGAGATTC	PASS	0	991	GGAGATTC	GGAGATTC	AGAGATTCCTCTCTG	TP53	NM_00054	TP53:exonic:NM_000546.5	missense	AAG	8	p.Glu285Lys	c.853G>A
chr17:757:SNV	G	1	G/A	PASS	0.087955	1006	G=992,A=1	G=0.9861,A=	1.39	TP53	NM_00054	TP53:exonic:NM_000546.5	missense	TGT	8	p.Arg273Cys	c.817C>T
chr17:757:SNV	G	1	G/A	PASS	0.002676	2000	G=1967,A=	G=0.9835,A=	1.65	TP53	NM_00054	TP53:exonic:NM_000546.5	missense	CTC	5	p.Pro153Leu	c.458C>T
chr17:757:SNV	G	1	G/C	PASS	0	718	G=12,C=70	G=0.0167,C=	0.9833	TP53	NM_00054	TP53:exonic:NM_000546.5	missense	CGC	4	p.Pro72Arg	c.215C>G
chr18:4851:SNV	G	1	G/A	PASS	0.006821	644	G=622,A=2	G=0.9658,A=	3.42	SMAD4	NM_00535	SMAD4:exonic:NM_005359.5	missense	GAA	5	p.Gly168Glu	c.503G>A
chr18:4851:SNV	C	1	C/T	PASS	0.006821	644	C=622,T=2	C=0.9658,T=	3.42	SMAD4	NM_00535	SMAD4:exonic:NM_005359.5	missense	TGT	5	p.Arg189Cys	c.565C>T
chr18:4851:SNV	C	1	C/T	PASS	0.023521	725	C=703,T=2	C=0.9697,T=	3.03	SMAD4	NM_00535	SMAD4:exonic:NM_005359.5	missense	CTT	9	p.Pro346Leu	c.1037C>T
chr19:179:SNV	C	1	C/T	PASS	0	1267	C=649,T=6	C=0.5122,T=	48.78	JAK3	NM_00021	JAK3:exonic:NM_000215.3	missense	ATC	16	p.Val722Ile	c.2164G>A



Cell analysis – Next generation Sequencing (NGS)

Sample Cancer Type: Unknown Primary Origin

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Clinically Significant Biomarkers

Genomic Alteration	Relevant Therapies (in this cancer type)	Relevant Therapies (in other cancer type)	Clinical Trials
<i>P TEN E256fs</i> phosphatase and tensin homolog	None	None	27
<i>TP53 M246V</i> tumor protein p53	None	None	8
<i>NOTCH1 V1578del</i> notch 1	None	None	1

Sources included in relevant therapies: FDA¹, NCCN, EMA², ESMO

Variant Details

DNA Sequence Variants

Gene	Amino Acid Change	Coding	Variant ID	Locus	Allele Frequency	Transcript	Variant Effect
NOTCH1	p.(V1578del)	c.4732_4734delGTG	COSM13047	chr9:139399408	2.14%	NM_017617.4	nonframeshift Deletion
PTEN	p.(E256fs)	c.767_770delAGTT	.	chr10:89717741	32.00%	NM_000314.6	frameshift Deletion
TP53	p.(M246V)	c.736A>G	COSM43555	chr17:7577545	31.17%	NM_000046.5	missense
FGFR3	p.(+)	c.1953G>A	.	chr4:1807894	98.64%	NM_000142.4	synonymous
PDGFR	p.(+)	c.1701A>G	.	chr4:55141055	99.83%	NM_006206.5	synonymous
KIT	p.(+)	c.1638A>G	COSM21983	chr4:55593481	47.87%	NM_000222.2	synonymous
APC	p.(+)	c.4479G>A	.	chr5:511217570	54.01%	NM_000038.5	synonymous
EGFR	p.(+)	c.2361G>A	.	chr7:55249063	100.00%	NM_005228.4	synonymous
RET	p.(+)	c.2307G>T	.	chr10:43613843	66.43%	NM_020975.4	synonymous
TP53	p.(P72R)	c.215C>G	.	chr17:7579472	38.86%	NM_000045.6	missense

Relevant Therapy Summary

 In this cancer type
  In other cancer type
  In this cancer type and other cancer types
  Contraindicated
  Both for use and contraindicated
  No evidence

PTEN E256fs

Relevant Therapy	FDA	NCCN	EMA	ESMO	Clinical Trials
capiwasertib + olaparib	✗	✗	✗	✗	● (II)
copanlisib	✗	✗	✗	✗	● (II)
everolimus	✗	✗	✗	✗	● (II)
niraparib	✗	✗	✗	✗	● (II)
talazoparib	✗	✗	✗	✗	● (II)
temsirolimus	✗	✗	✗	✗	● (II)
atezolizumab + ipatasertib	✗	✗	✗	✗	● (I/II)
ARQ-751	✗	✗	✗	✗	● (I)
AZD8186 + chemotherapy	✗	✗	✗	✗	● (I)
AZD8186, AZD8186 + abiraterone acetate + steroid, AZD8186 + vistusertib	✗	✗	✗	✗	● (I)
gedatolisib + palbociclib	✗	✗	✗	✗	● (I)
palbociclib + pictlisib, palbociclib + taselisib	✗	✗	✗	✗	● (I)
ipatasertib + chemotherapy	✗	✗	✗	✗	○ (III)
abemaciclib + LY-3023414	✗	✗	✗	✗	○ (II)
alpelisib	✗	✗	✗	✗	○ (II)

Current Clinical Trials Information

Clinical Trials information is current as of 2019-03-01. For the most up-to-date information regarding a particular trial, search www.clinicaltrials.gov by NCT ID or search local clinical trials authority website by local identifier listed in 'Other identifiers'.

PTEN E256fs + TP53 M246V

NCT03263650 Randomized Phase II Study of Olaparib Maintenance Following Carbixatxel-Carboxatin Induction Chemotherapy in Men With Aggressive Variant Prostate Cancer (AVPC) Cancer type: Prostate Cancer Variant classes: PTEN & TP53 mutation	Other identifiers: 2017-01333, NCI-2018-01701 Population segments: Hormone refractory, Maintenance/Consolidation, Stage IV Phase: II Therapy: olaparib Location: United States US State: TX Contact: Dr. Ana M. Aparicio [713-792-2830; aaparcio@mdanderson.org]
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NCT03207347

A Phase II Trial of the PARP Inhibitor,
Niraparib, in BAP1 and Other DNA
Damage Response (DDR) Pathway
Deficient Neoplasms (UF-STO-ETI-001)

Cancer type: Unspecified Solid Tumor
Variant class: PTEN mutation

Other identifier: UF-STO-ETI-001

Population segments: (N/A), Second line

Exclusion criteria variant classes: ABRAXAS1 mutation (Prostate Cancer), ARID1A mutation (Prostate Cancer), ATM mutation (Prostate Cancer), ATR mutation (Prostate Cancer), ATRX mutation (Prostate Cancer), BAP1 mutation (Prostate Cancer), BARD1 mutation (Prostate Cancer), BLM mutation (Prostate Cancer), BRP1 mutation (Prostate Cancer), CDK4 mutation (Prostate Cancer), CHEK1 mutation (Prostate Cancer), CHEK2 mutation (Prostate Cancer), ERCC mutation (Prostate Cancer), IDH1 mutation (Prostate Cancer), IDH2 mutation (Prostate Cancer), MRE11 mutation (Prostate Cancer), NBN mutation (Prostate Cancer), PALB2 mutation (Prostate Cancer), POLD1 mutation (Prostate Cancer), PRKDC mutation (Prostate Cancer), PTEN mutation (Prostate Cancer), PRKDC mutation (Prostate Cancer), RRM1 mutation (Prostate Cancer), SLX4 mutation (Prostate Cancer), WRN mutation (Prostate Cancer), XRCC mutation (Prostate Cancer)

Phase: II

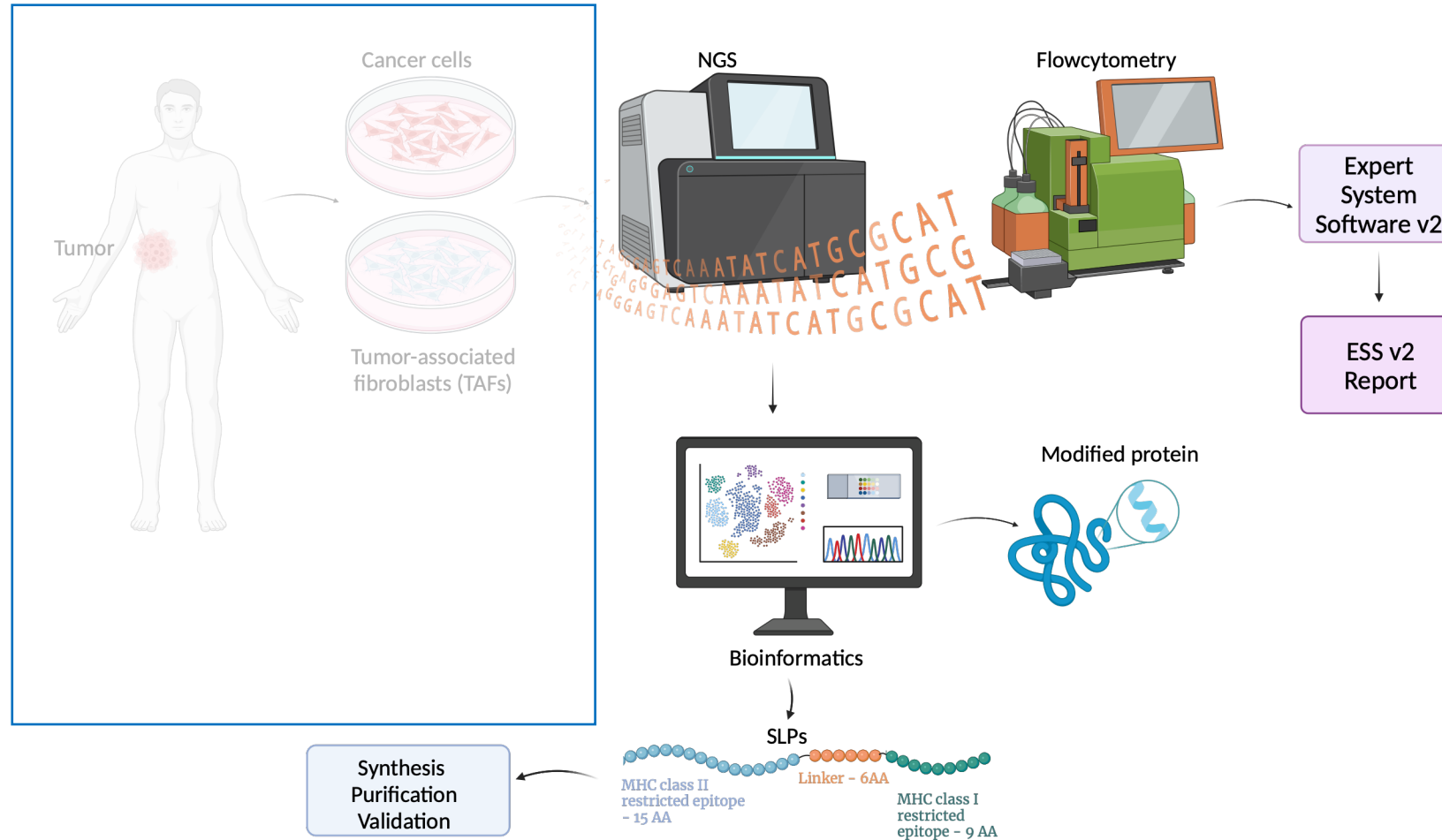
Therapy: niraparib

Location: United States

US State: FL

Contact: Ashton Monismith [352-265-0680 ext 87657; amonismith@ufl.edu]

Next steps



Timeline

BETTER

diagnosis,
care,
lives!



Thank you!