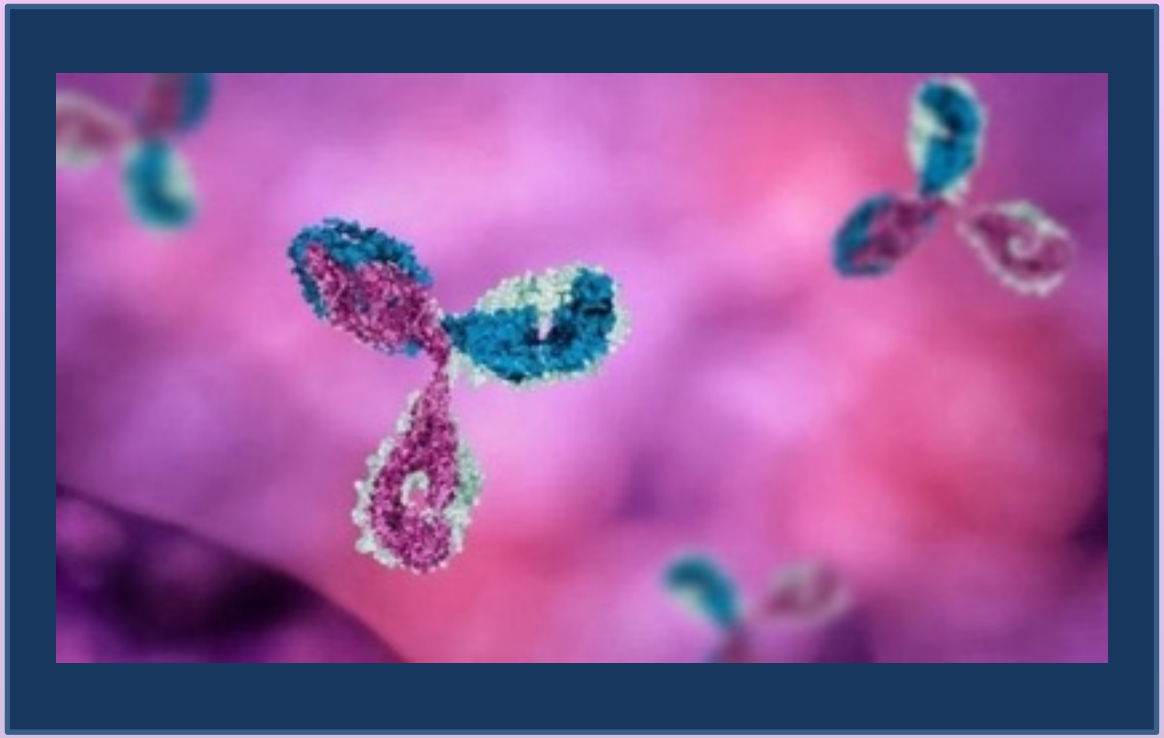
Training school on Autoimmune Neutropenias 2020/2021

GIFT
Granulocyte Immunofluorence Test



Francesca Fioredda
Hematology Unit
IRCCS Giannina Gaslini Hospital

EuNet INNOCHRON

ANTIGENS' COMPLEXITY

Table 1
Subsets of immature and mature neutrophils in the bone marrow.

	Nuclear morphology of immature and mature mouse neutrophils	Surface markers in Human	Surface markers in Mouse
Granulocyte monocyte progenitor (GMP)		Lin^-, $CD34^+$, $CD38^+$, $CD45RA^+$, $CXCR4^+$, $CXCR2^-$	Lin^-, $Sca1^-$, $CD117^{hi}$, $CD11b^-$, $CD34^+$, $CD16/32^+$, $CXCR4^+$, $CXCR2^-$, $Gr1^-$, $Ly6C^-$
Early committed neutrophil progenitor1 (proNeu1)		$CD49d^{hi}$, $CD34^+$, $CD61^{hi}$, $CD11b^-$, $CD38^{hi}$, $CD24^{lo}$	Lin^-, $CD115^-$, $Flt3^-$, $Ly6C^+$, $CD117^{hi}$, $CD34^+$, $CD16/32^+$, $CD106^-$, $CD11b^{lo}$,
Early committed neutrophil progenitor2 (proNeu2)		$CD49d^{int}$, $CD34^-$, $CD61^{lo}$, $CD11b^-$, $CD38^{lo}$, $CD24^{hi}$	Lin^-, $CD115^-$, $Flt3^-$, $Ly6C^+$, $CD117^{hi}$, $CD34^+$, $CD16/32^+$, $CD106^+$, $CD11b^{hi}$
Committed proliferative neutrophil precursor (preNeu)		Lin^-, $Siglec8^-$, $CD15^+$, $CD34^-$, $CD66b^+$, $CD101^-$, $CD49d^{lo}$, $CD61^{lo}$, $CD11b^{lo}$, $CXCR4^+$, $CXCR2^-$	Lin^-, $CD115^-$, $Siglec\text{-}F^-$, $CD117^{int}$, $Gr1^+$, $CD11b^+$, $Ly6G^{lo}$, $CXCR4^{hi}$, $CXCR2^-$
Band Neutrophil		$Siglec8^-$, $CD66b^+$, $CD15^+$, $CD33^{mid}$, $CD49d^-$, $CD10^-$, $CD16^{low}$, $CD101^+$, $CD34^-$, $CD61^{lo}$, $CD11b^{lo}$, $CXCR4^+$, $CXCR2^+$	Lin^-, $CD115^-$, $Siglec\text{-}F^-$, $CD117^-$, $Gr1^+$, $CD11b^+$, $CD101^-$, $Ly6G^{lo/int}$, $CXCR4^{lo}$, $CXCR2^-$
Mature Neutrophil		$Siglec8^-$, $CD66b^+$, $CD15+$, $CD49d^-$, $CD10^+$, $CD101^+$, $CD16^{hi}$, $CD34^-$, $CD61^{lo}$, $CD11b^{hi}$, $CXCR4^-$, $CXCR2^+$	Lin^-, $CD115^-$, $Siglec\text{-}F^-$, $CD117^-$, $Gr1^+$, $CD11b^+$, $CD101^+$, $Ly6G^{hi}$, $CXCR4^-$, $CXCR2^+$

Carnevale S, Sem Immun 2020

Type of antibodies

System	Glycoprotein/Gene	Allele	Epitope(s)
HNA-1 (NA1, NA2, SH)	CD16b/*FcγRIIIb*	*FCGR3B**01	HNA-1a
		*FCGR3B**02	HNA-1b, HNA-1d
		*FCGR3B**03	HNA-1b, HNA-1c
		*FCGR3B**04	HNA-1a
		*FCGR3B**05	HNA-1b variant
	No Glycoprotein	*FCGR3B**null	HNA-1 null
HNA-2 (NB1)	CD177	CD177[a]	HNA-2
	No Glycoprotein	No allele	HNA-2 null
HNA-3 (5b, 5a)[b]	CLT2/*SLC44A2*	*SLC44A2**01	HNA-3a
		*SLC44A2**02	HNA-3b
		*SLC44A2**03	HNA-3a variant
HNA-4 (Mart)	CD11b.CD18/*ITGAM*	*ITGAM**01	HNA-4a
		*ITGAM**02	HNA-4b
HNA-5 (Ond)	CD11a.CD18/*ITGAL*	*ITGAL**01	HNA-5a
		*ITGAL**02	(HNA-5bw)[c]

Detection of Antibodies Against Neutrophils

Detection of antibodies against surface antigens

- ✓ Radiolabeled (anti IgG, Protein A, Anti IgM)
- ✓ Intact Staphylococci
- ✓ Immunofluorescence
- ✓ Flow cytometry
- ✓ Enzyme-linked immunoassays

GIFT

Detection of antibodies effect

- ✓ Agglutination Opsonization
- ✓ Complement Activation
  - antibodies to C3
  - cytotoxicity
- ✓ Antibody dependent cytotoxicity

GAT

Shastri KA , Blood 1993

Work with Neutrophils

Obstacles

- Neutrophils fragility
- Tendency to aggregation in vitro
- Release of autolytic enzymes on handling (contact with glass, temperature, centrifugation)
- Autoactivation and «fake autommunity»
- Decreased number of neutrophils

METHODS TO DETECT ANTIBODIES

evolution

GAT
GIFT/LIFT
GCLT
MAIGA
rHNA 3a/3b
Fluorescente beads

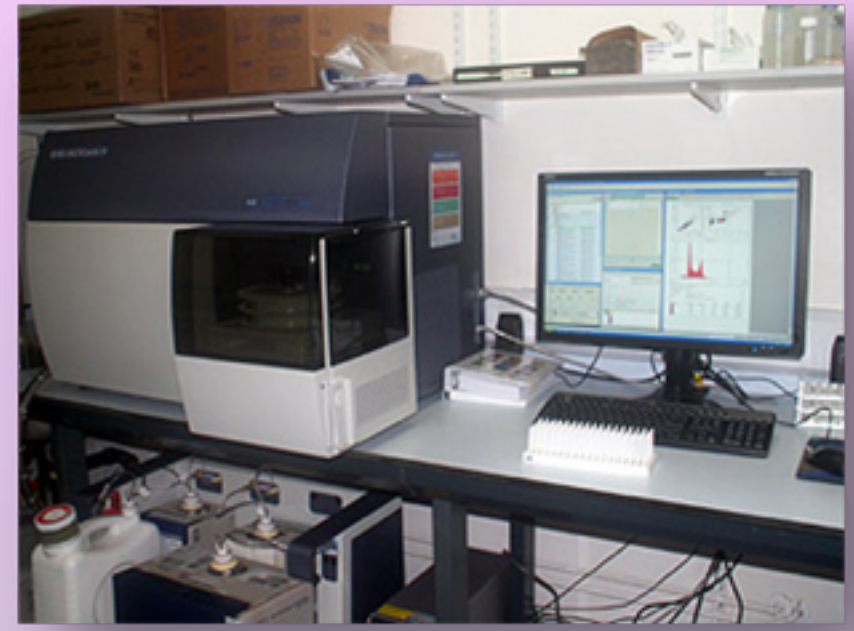
The combination of GIFT and GAT guarantees a standard approach
International Granulocyte Immunology Workshop

Flesh B Vox sanguines 2019 ,Lucas G, Vox Sanguinis 2013, Browne T Int J Immunogenet 2020

GIFT

Flow cytometry

Precise measurement of several characteristic of particulate cells



Monoclonal Antibodies allows the recognition of Cluster Designation (CD)

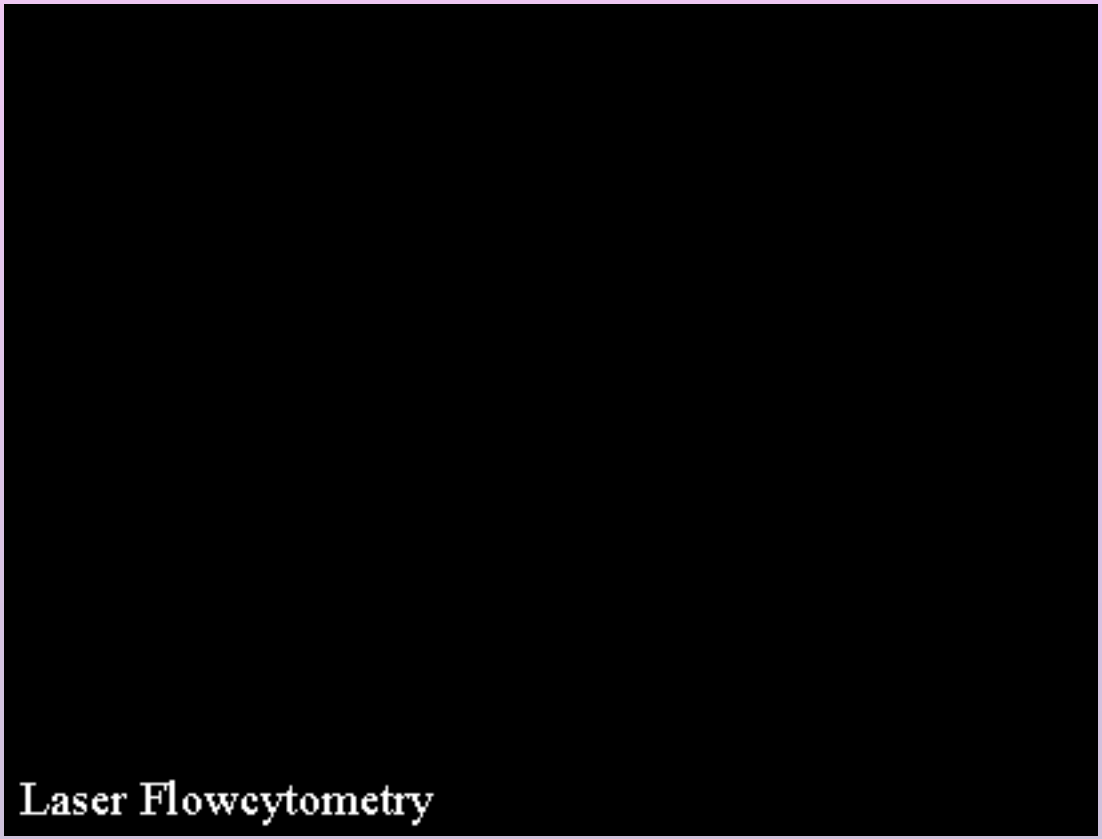
MO Ab against FcR

MO Abs labeled wit fluorochrome FITC or PE

Carulli G Hem Mol Hematol 1996

Basic Flow principles

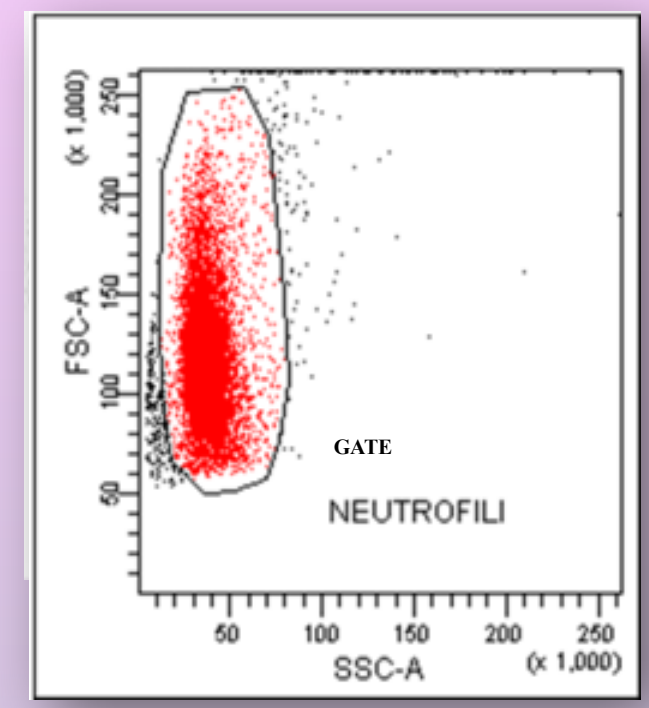
FLOW CYTOMETRY / FACS SCanto



Basic Flow principles

Complexity

SSC degree scatter



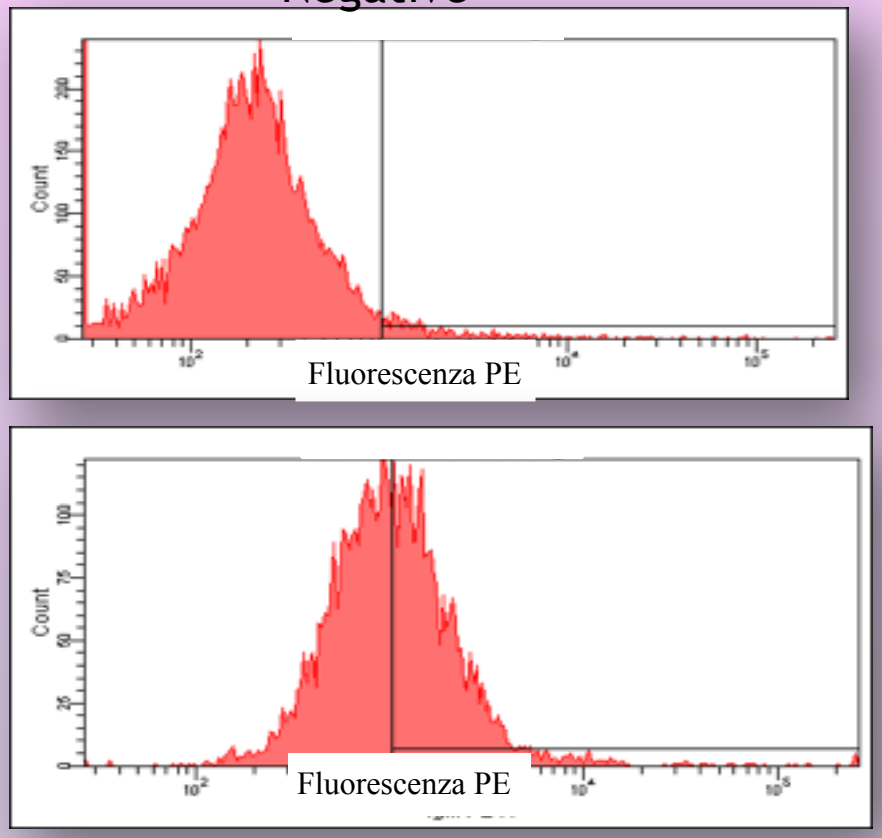
FSC forward scatter

Volume

Basic Flow principles

MO Abs labeled wit fluorochrome FITC or PE

Negative



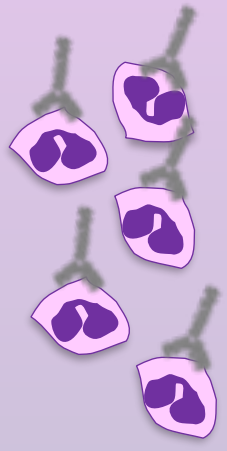
Positive

Indirect GIFT

immunofluorescence test (GIFT)

Direct GIFT

immunofluorescence test (GIFT)



Indirect GIFT

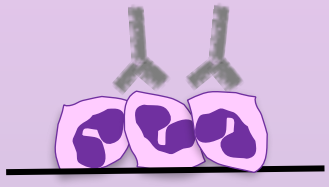
immunofluorescence test (GIFT)

Blood samples

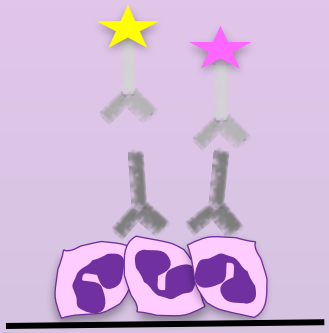
Pooled neutrophils
from healthy donors

Patient serum

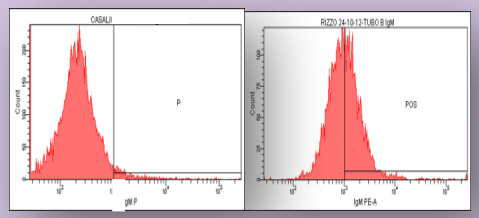
Antigen/antibody reaction



Fluorescent staining



Reading by Flow



I-GIFT

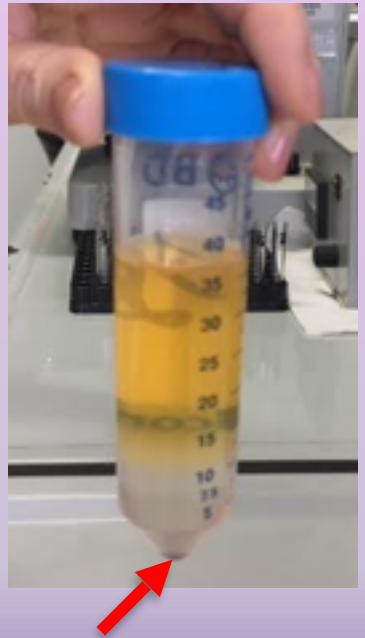
THE TECNIQUE 1

SERUM COLLECTION centrifugation and conservation at -20°C

SAMPLE PREPARATION

1) Anticoagulated whole blood coming from a pool of healthy donors (males, preferably 0 Rh) addicted with 6% dextran for 30 ‘ at 37°C

2) Separation by Fycoll gradient: centrifugation and collection of neutrophils which stand in the bottom Washing with PBS



3) Addition of lysis buffer 10’ incubation and centrifugation

Neutrophils ~97% of the product

I-GIFT
THE TECNIQUE 2

ADESION OF ANTIBODIES

Neutrophils incubated with patient/control serum 1:1 for 20’

STAINING

Cells washed twice then incubated at 37°C with Goat F (ab’) anti human IgG-PE or Goat F(Ab’) and IgM-PE

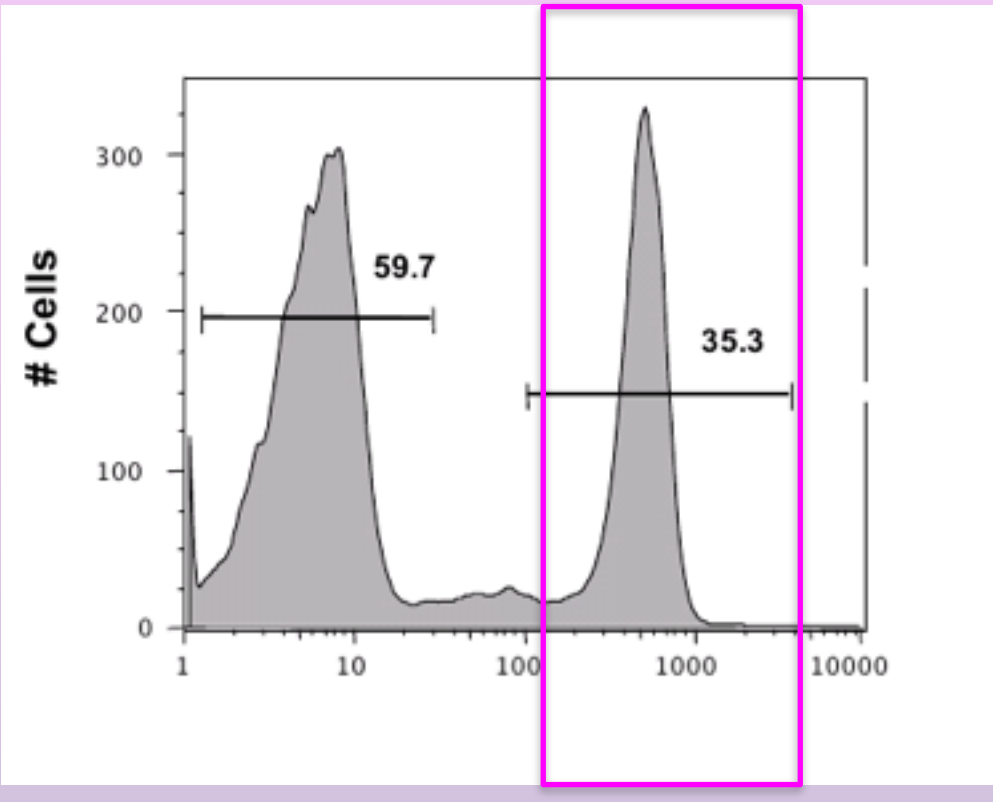
I-GIFT
THE TECNIQUE 3

FLOW CYTOMETRY / FACS SCanto

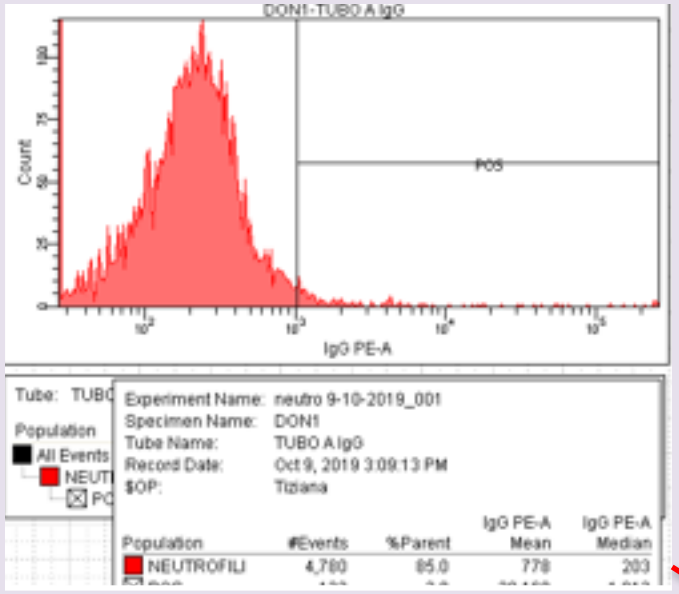
- Intensity of IgG and IgM binding to the neutr Ag is masured by Mean Fluorescence Intensity (MFI)
- Positive serum: MFI 2 SD > than the mean derived by over 100 normal control sera for each Ab (IgG and IgM)

Basic Flow principles

Results expressed as computer generated histograms of percentage of highly fluorescent cells



Flow read out IgG- Donor

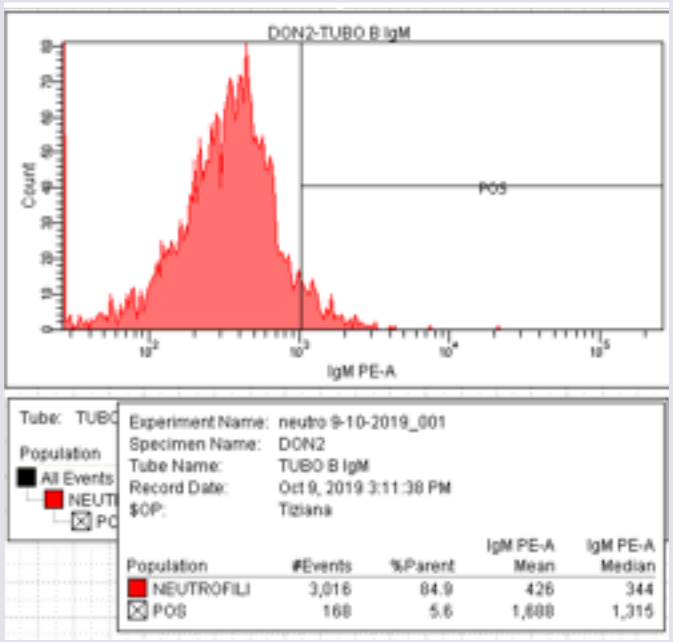


IgG= our lab MFI+ 2SD=327

MFI = 203

NEGATIVE SAMPLE

Flow read out IgM- Donor



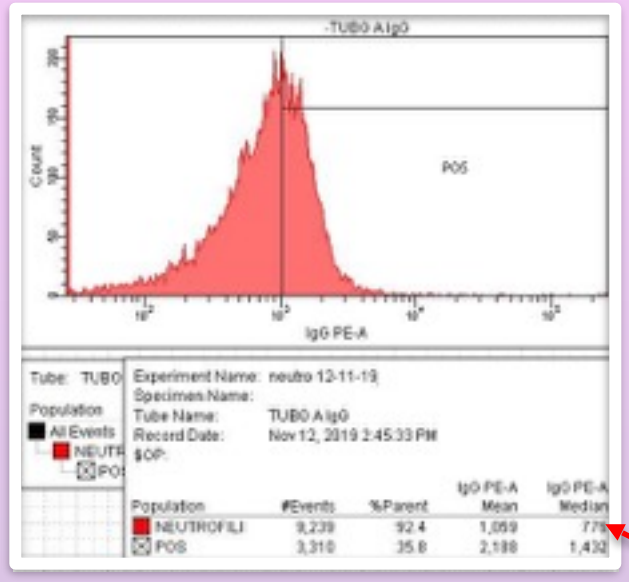
MFI = 344

IgM, our lab MFI+ 2SD = 519

NEGATIVE SAMPLE

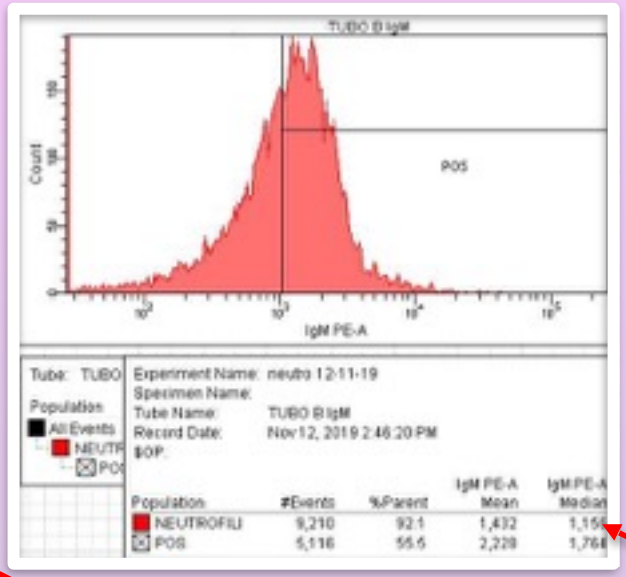
Flow read out patient

IgG positive pz



MFI = 776

IgM positive pz



MFI = 1156

IgG= our lab MFI+ 2SD = 327

POSITIVE SAMPLE

IgM, our lab MFI + 2SD = 519

POSITIVE SAMPLE

I-GIFT

Immunofluorescence test (GIFT)
Contribution of IgM detection

180 samples

19% IgG
21% IgG and IgM
8% IgM

IgM detection increases sensitivity of the test

Hartmann KR American J of Med Science 1994

SENSITIVITY

First Author	No pts	Age at diagnosis (months)	Sensitivity with one test
Lalezari P 1986	121	8 (3-30)	95%
Bux J 1998	240	8 (5-15)	74%
RutiSella BA 2010	72	10 (0-42)	62.5%
Bruin M 1999	21	<12 mo	Only positive
Audrain M 2011	116	16 (3-59)	60%
Wang L 2009	55	9.8 (4-28)	74%
Farruggia P 2015	157	8 (range 0–54):	62%

in 26%
> by the 3/4 th

82%
by the 4th

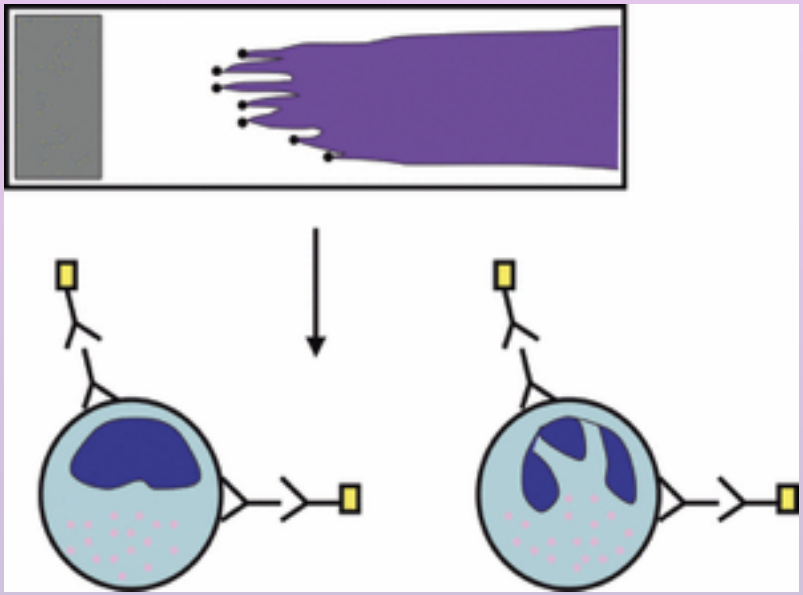
Bux J , Blood 1998, Audrain M Ped All Immunol 2011, Brun MC Blood 1999 , Chung B Hong Kong Med J 2004, Wang L Transfusion 2009, Lalezari J Ped 1986, Ruti Sella IMAJ 2010 , Farruggia P Am J Hem 2015

I-GIFT

immunofluorescence test (GIFT)

Bone marrow

BMIFT 24/45 samples sensitivity 55% , specificity 100%

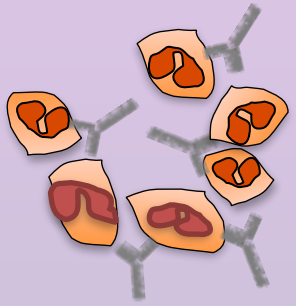


Lane SW Int Jnl Lab Hem 2009

Direct-GIFT

immunofluorescence test (GIFT)

- Whole blood incubated on ice with FITC- conjugated anti human IgG +Mo
- Disruption RBC
- Flow Cytometry read-out



Ito T J Ped Hematol Oncol 2011 , Porretti L Ped Blood and Cancer 2018

GIFT

immunofluorescence test (GIFT)

Cons

Direct test

Low numbers of neutrophils

Spontanoeus activation of neutrophils

High n° false positive (ICC)

Need for short sampling – test interval

Indirect test

High rate of false negative

Possible false positive

Bux J , Blood 1998, Taniuchi S J p Ped Hematol Oncol 2002 , Porretti L Ped Blood and Cancer 2018 , Farruggia P Worl J 2015, Capsoni F

GIFT

immunofluorescence test (GIFT)

Pros

Direct test

Reported good sensitivity

Need low quantity of blood

Indirect test

High Predictive Positive Value

Easy centralization of samples

Bux J , Blood 1998, Taniuchi S J p Ped Hematol Oncol 2002 , Porretti L Ped Blood and Cancer 2018 , Farruggia P Worl J 2015, Capsoni F

TAILORING THE STRATEGY

GIFT Improves sensibility on repeated testings

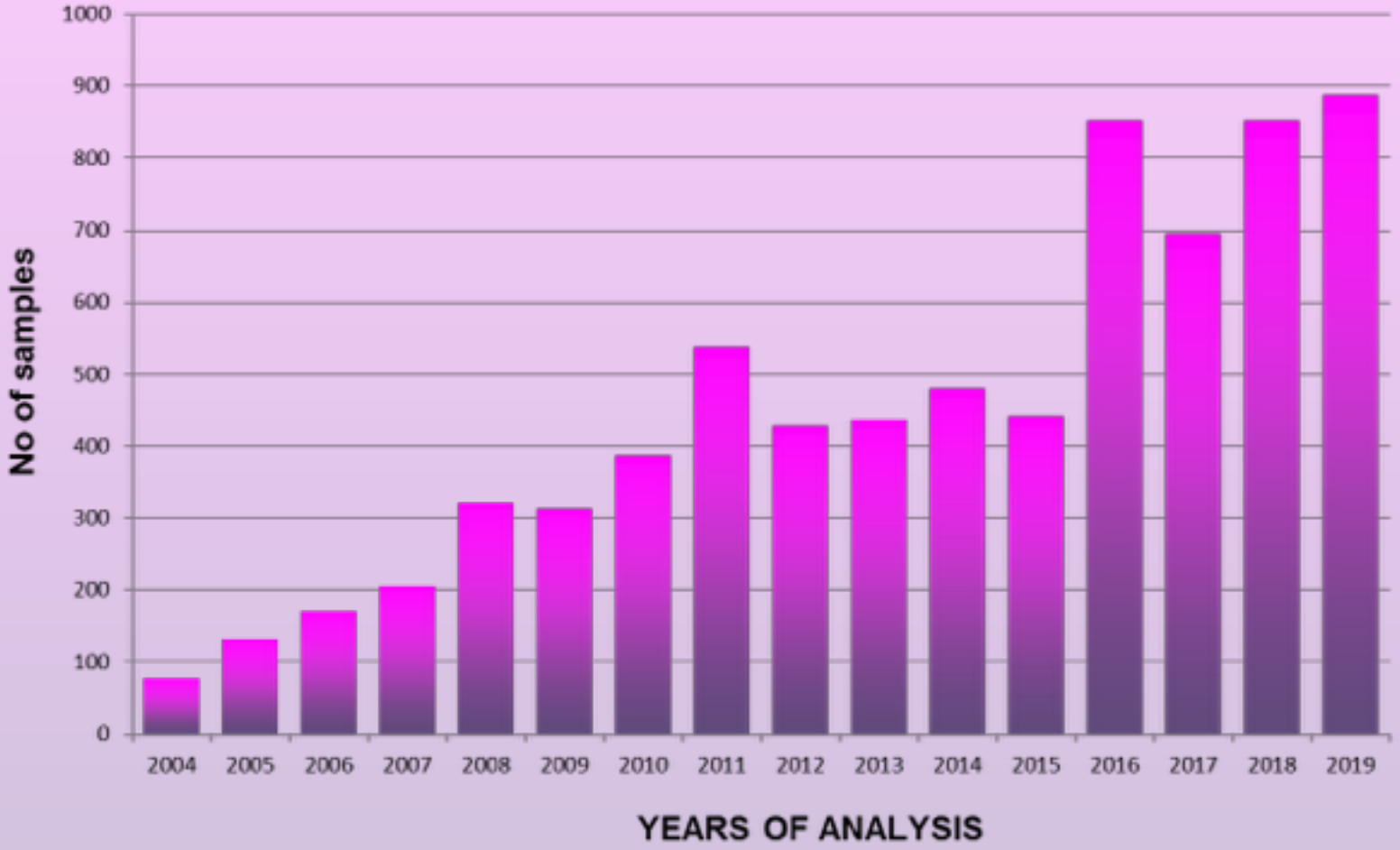
GIFT is suitable for centralized analysis of large number of samples

AIEOP centers

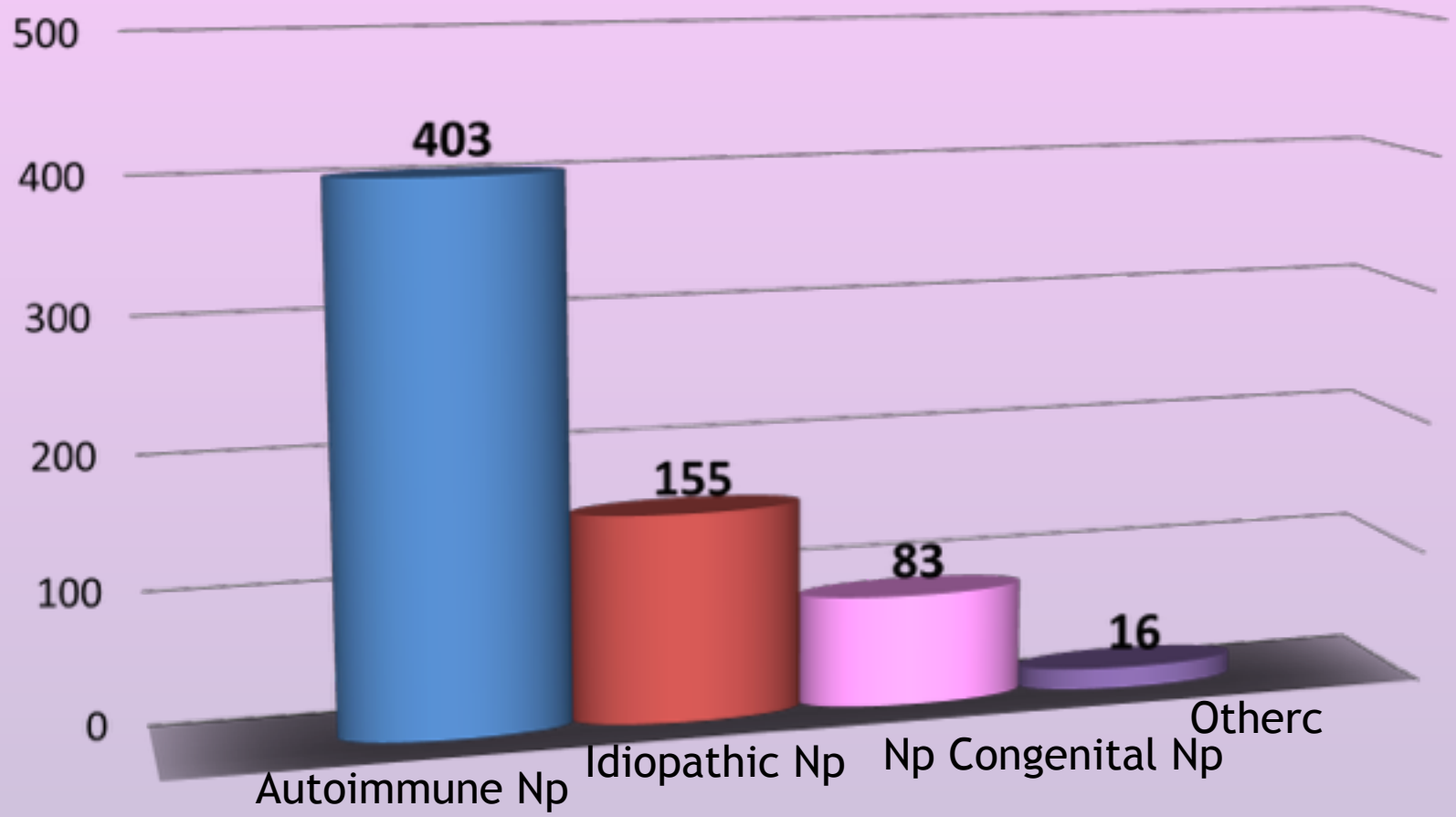
Gaslini Laboratory

Italian Reference Lab

SAMPLES FLUX – Unit of Hematology
IRCCS Gaslini Italian Reference Lab



ITALIAN NEUTROPENIA REGISTRY
2020 Update



FINAL RECCOMANDATION

Diagnosis of Autoimmune Neutropenia in Child

I-GIFT

repeated 3-4 times

if negative

Fioredda F Pediatr Blood &Cancer 2011

THANKS!

Polo di Emato-Oncologia-TMO IRCCS

Carlo Dufour
Concetta Micalizzi
Elena Palmisani
Michaela Calvillo
Maurizio Miano

Daniela Guardo
Erika Massaccesi

Tiziana Lanza

Paola Terranova
Marina Lanciotti