Gating procedure for white blood cells (WBC) on NAVIOS

The French PNH diagnosis working group has developed an optimized gating strategy adapted to a one-tube, six-colour diagnostic test, evaluated in a multicentric study.
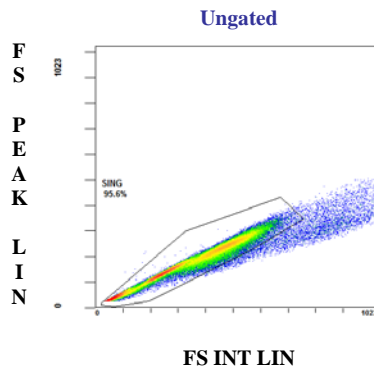The combination includes CD45, CD15 and CD33 as gating markers and CD24, CD14 and FLAER as diagnostic markers.
The gating procedure is composed of three parts:
- Firstly, the cell populations of interest among the leukocyte subtypes (polymorphonuclear and monocytes) are gated based on their level of CD45, CD15 and CD33 expression.
- Secondly, the presence of a type III PNH clone is assessed using internal control cell populations.
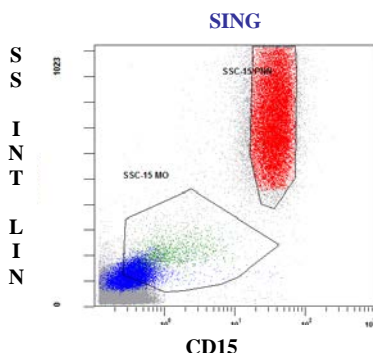- Finally, the precise size of the clone is evaluated.

Identifying WBC subsets: polymorphonuclear (PMN) and monocytes (MO)

On FSC-A/FSC-H plot-plot: Exclusion of the doublets display total events

Adjust the region "SING" to include single events.

Check on the density plot.

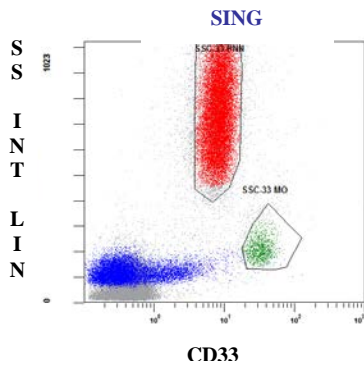## On CD15/SSC plot: display single cells

Adjust the SSC-15 PMN region to include CD15 positive events, regardless of their level of SSC intensity. Do not include events with low CD15/high SSC intensity, so that immature granulocytes and eosinophils are excluded.

Adjust the SSC-15 MO region to include low-intensity or CD15 negative events and intermediate SSC intensity, without overlapping with the SSC-15 PMN region.
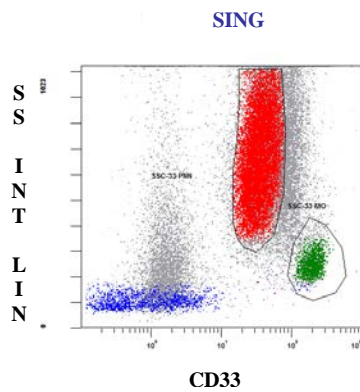
On CD33/SSC plot: display single cells

Normal sample :

Adjust the SSC-33 MO region to include events with bright CD33 intensity and low SSC intensity. Do not include events with intermediate CD33 intensity and low SSC intensity – this population contains basophils and dendritic cells.
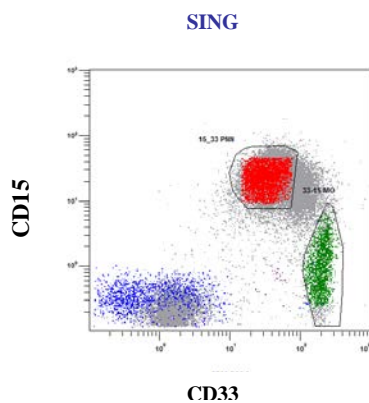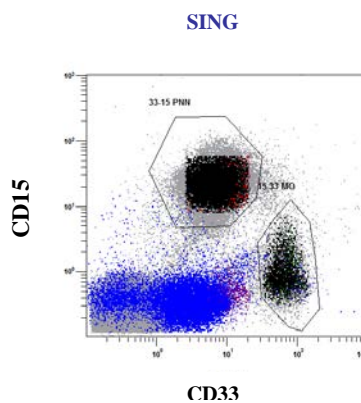
Immature granulocytes

Adjust the SSC-33 PMN region to include events with intermediate to low CD33 intensity, whilst excluding events with low SSC intensity. Do not include events with bright CD33 intensity containing immature granulocytes (purple).

On CD33/CD15 plot : display single cells
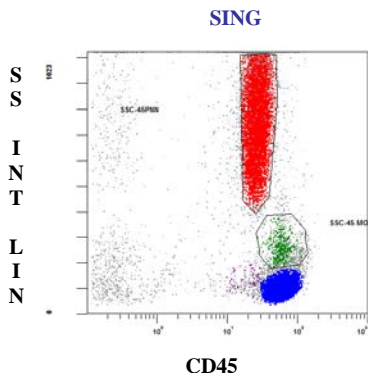
Immature granulocytes :   PNH sample:

Adjust the 33-15 MO region to include events with bright CD33 intensity.

Adjust the 33-15 PMN region to include CD15-positive events and events with intermediate CD33 intensity.
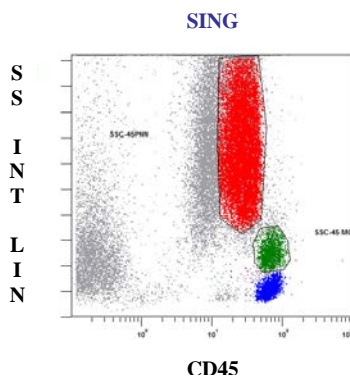
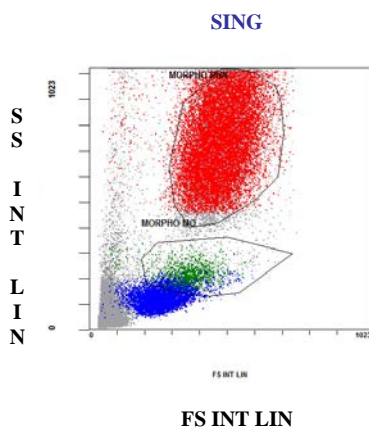## On CD45/SSC plot : display single cells

Normal sample :

Adjust the SSC-45 PMN region to include events with intermediate CD45 intensity. Do not include events with low SSC intensity (blasts and basophils), events with intermediate CD45 intensity containing immature granulocytes, and events with high CD45 intensity and high SSC intensity containing eosinophils.

Immature granulocytes :

On FSC/SSC plot: display the intersection of the 3 regions SSC-15, SSC-33 and 15-33 for PMN and monocytes, respectively

Normal sample:

Adjust the MORPHO PMN and MORPHO MO regions to exclude dead cells. Eosinophils are mostly excluded but not immature granulocytes.

Identifying a PNH type III population using control cell populations

The aim of this step is to assess either the presence of PNH type III cells or the absence of events with a full defect in GPI-linked proteins.
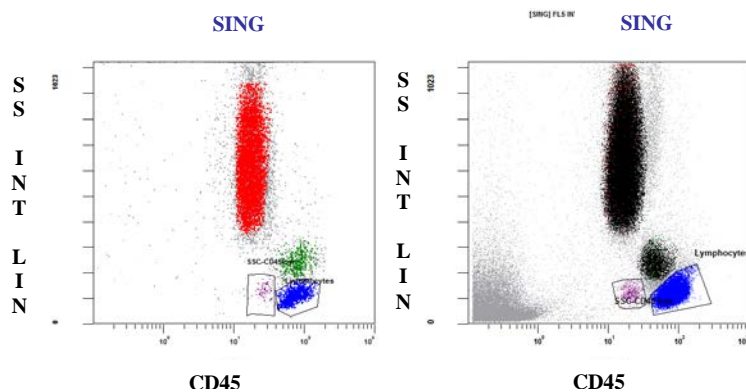
This approach consists in using internal control cell populations to define the threshold of positivity of the diagnostic markers.

The populations used are total lymphocytes, B-lymphocytes and basophils.

On CD45/SSC plot: display single cells
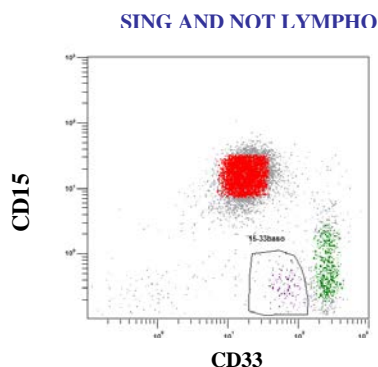
Normal sample: PNH sample:

Adjust the LYMPHOCYTE region to include events with high CD45 intensity and low SSC intensity (blue).

Adjust the SSC CD45 low region to target basophils.

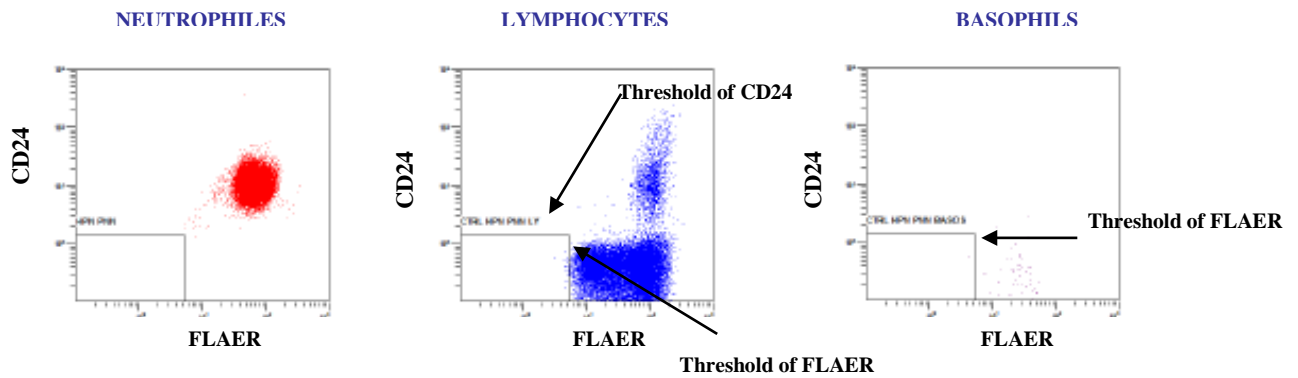**On CD33/15 plot**:   display single cells and not lymphocytes

Normal sample :

Identify basophils (dark purple) on events with intermediate CD33 intensity and CD15 negative events (15-33 baso region).

Next, the PNH area is set according to the expression of the diagnostic markers on the control populations.
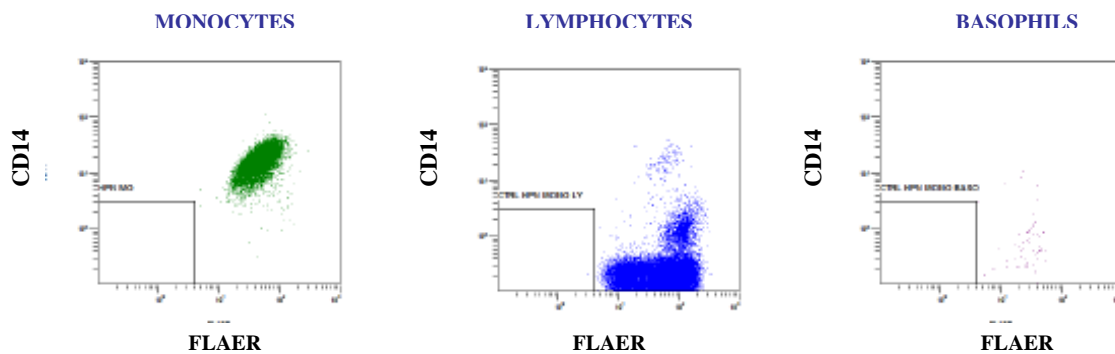
On FLAER/CD24 plot : determination of FLAER-CD24-region

The threshold of FLAER is set at the lowest expression of lymphocytes and/or basophils (positive control limit). Similarly, the threshold of CD24 is set using non-B-cell lymphocyte populations (CD24 negative) and/or basophils as positive controls.

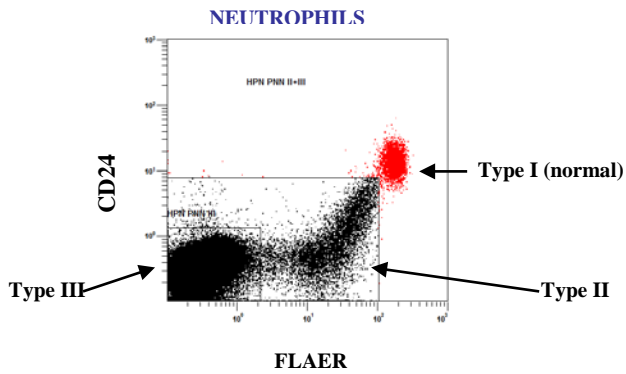**On FLAER/CD14 plot:** determination of FLAER-CD14-region

For the PNH region on monocytes, we use the same threshold for FLAER. Determination of the threshold of CD14 is set according to the lymphocytes.

Determination of the size of the PNH clone

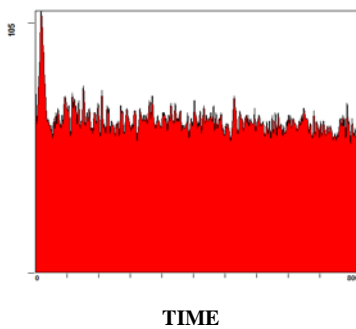## Optimization of the gating:

In this final step, the PNH area is optimized in order to include type II cells on the plots displaying PMN or monocytes.

In the presence of leukocytes with low expression of GPI-linked proteins (Type II cells), create a second region to determine the percentage of these cells.
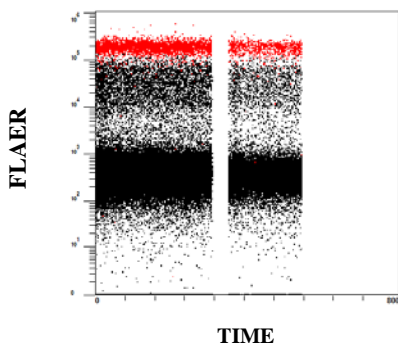
Finally, additional controls such as time plots can be used to ensure a reliable result.

Check flow during data acquisition: take note of any changes in speed or cell concentration (dilution, sedimentation, peak of events at the end of acquisition, occlusion ...).

Check the distribution of PNH cells during data acquisition. It is valid only if the number of PNH cells detected follows the same variations as normal neutrophils.