Compensation - Using Compensation Setup

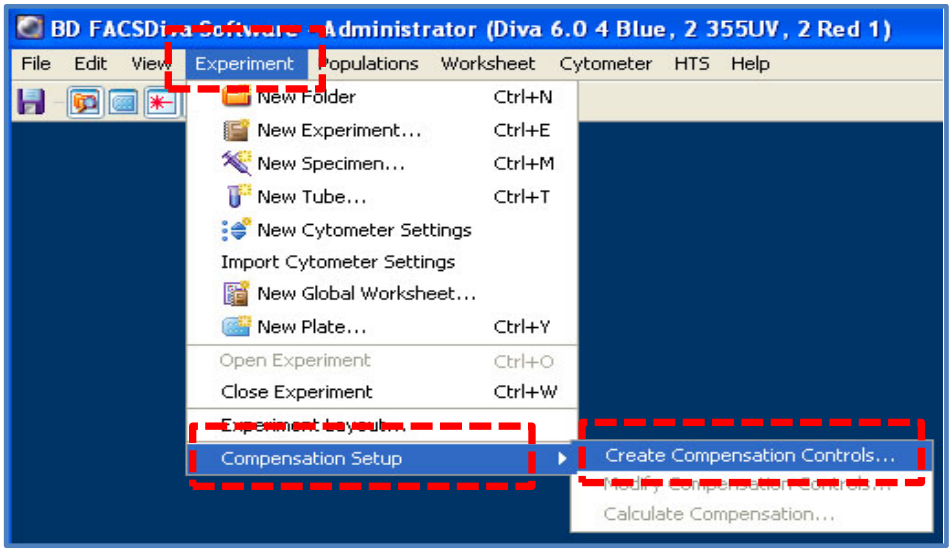
Compensation setup is designed to work with single-stained controls. These controls can consist of single-stained cells or capture beads. We recommend to prepare a separate tube with negative beads as your negative control.

Start to create the compensation controls…

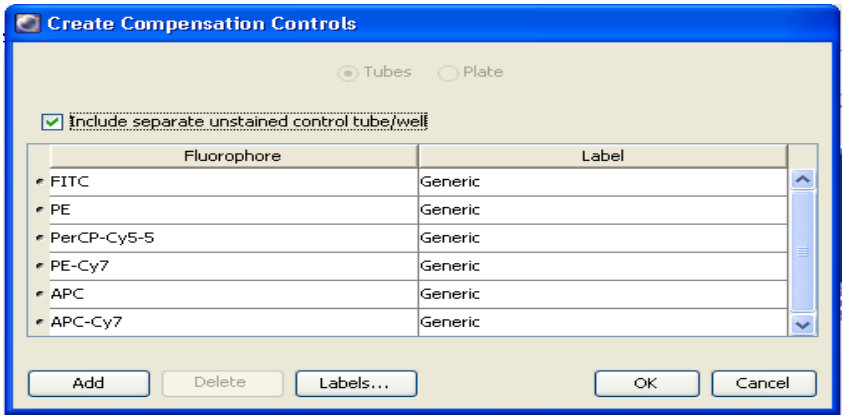
- **after your experiment is created (means all fluorochromes are added in the parameters list and no needed fluorochromes are deleted)**
- **after you check your negative control sample, set up FSC and SSC**

1. Creating Compensation Controls

- select **Experiment > Compensation Setup > Create Compensation Controls**

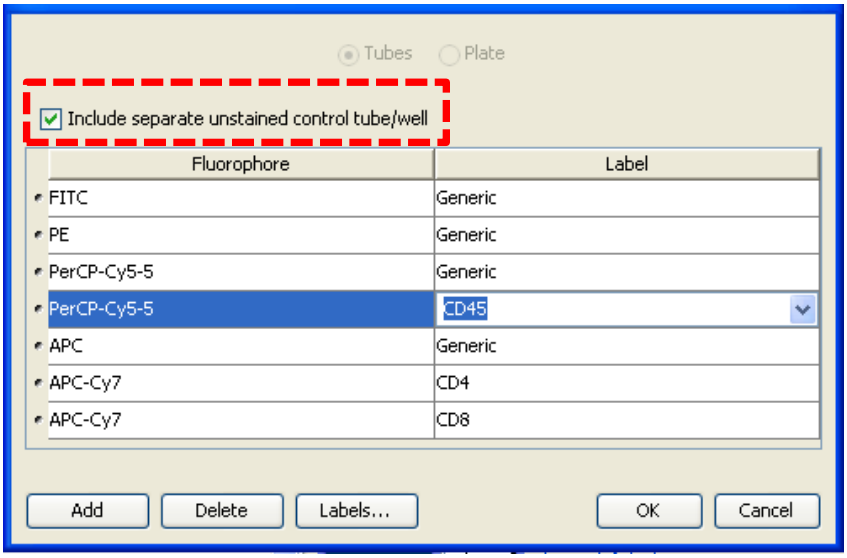


In the “Create Compensation Controls Panel”, you can add only parameters that are listed in the Parameters tab. To change to another fluorochrome for any parameter, edit cytometer settings in the Cytometer window or Inspector *before* you create compensation controls.

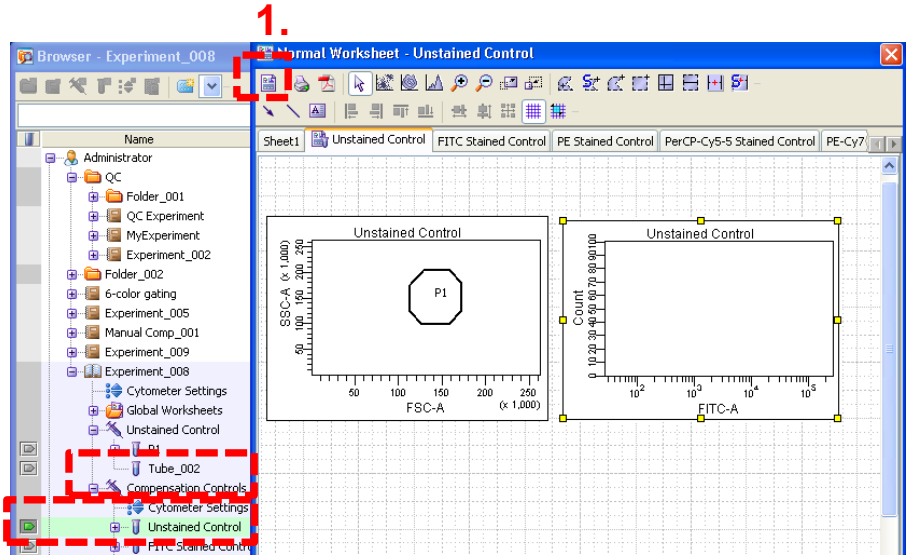


Legend:
- *Add*: add a Fluorophore
- *Delete*: delete a Fluorophore
- *Labeles..*: change the Generic lable into your own name

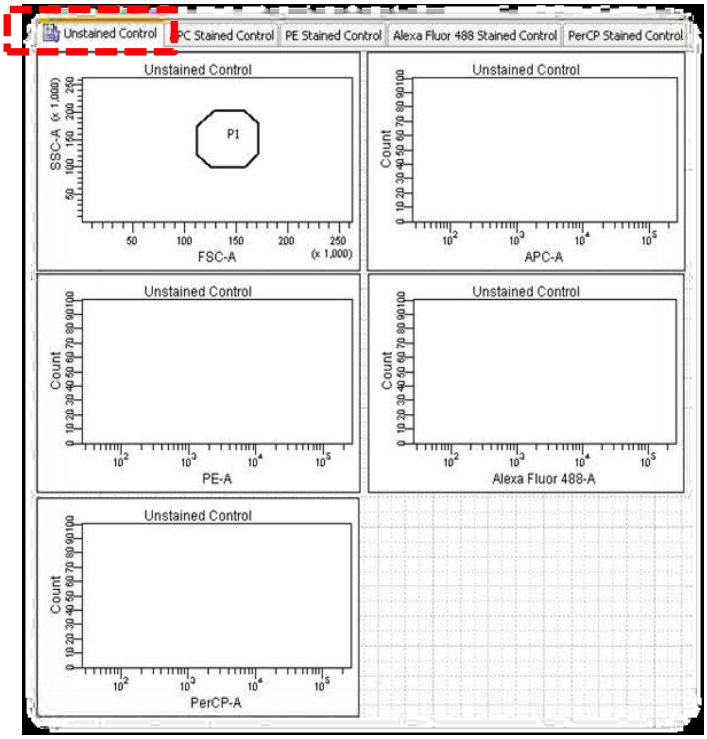
to define antigen-specific controls while creating or modifying compensation controls



- make sure that the **“Included separate unstained control tube”** is marked
- if you have labeled your fluorophores with your experiment specific antigens in the experiment layout, you'll find your label (CD or something else) **and** generic in the compensations control panel. **Delete generic ones**.
- if no label has been entered, measure under "Generic". Thus, not specifically assigned to an antigen, e.g. CD34 but to the associated fluorochrome.
- click **“OK”**
- a new specimen named **“Compensation Control”** is added and listed within your experiment
- the software automatically opens the compensation panel in the **“Normal Worksheet”**
- to return to your experiment **(“Global Worksheet”)** press the button marked with **1.**



- mark the compensation tube **“unstained control”** (**marked green**)
  the “Unstained Control” tube displays a histogram for each fluorochrome you are going to compensate



1.2.1 Setting the spillover values with a <u>separate negative control</u>

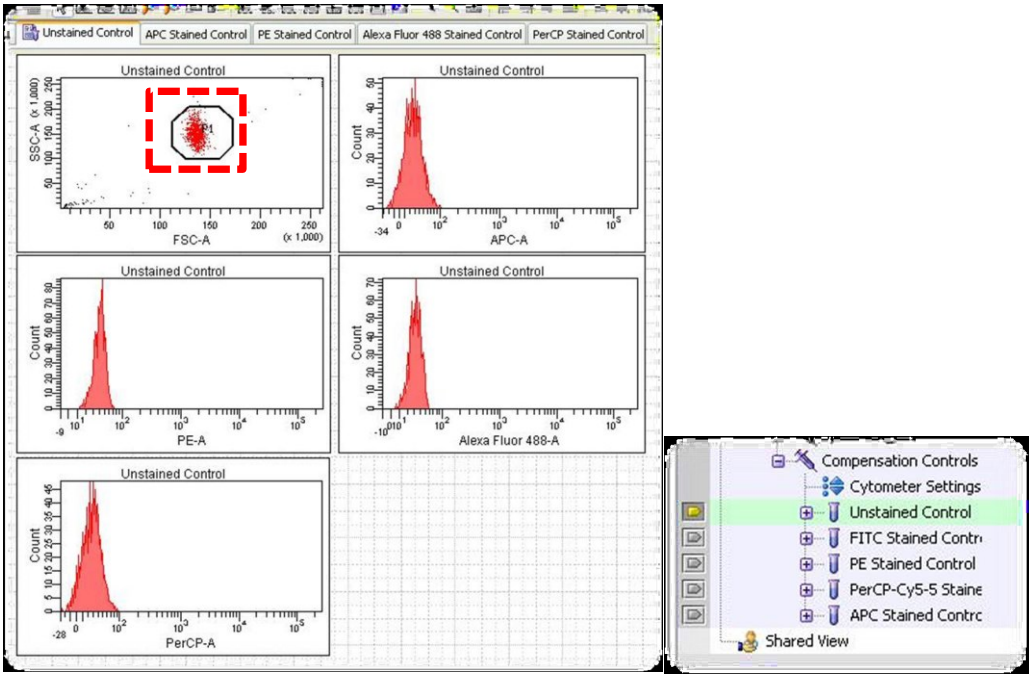
- **if you don´t use a separate negative control, you have to gate also the negative cells or beads in your compensation control - see page 6/7**

- mix your unstained control beads tube and load it to the **Sample Injection Port (SIP)** press **“Low”** and **“Run”**

- click **“Acquire Data”** and wait a few seconds until the threshold rate is stable

- adjust gate P1 to include your population of interest

- adjust P1 to all samples, right mouse tab > **“Apply to all Compensation Controls”**

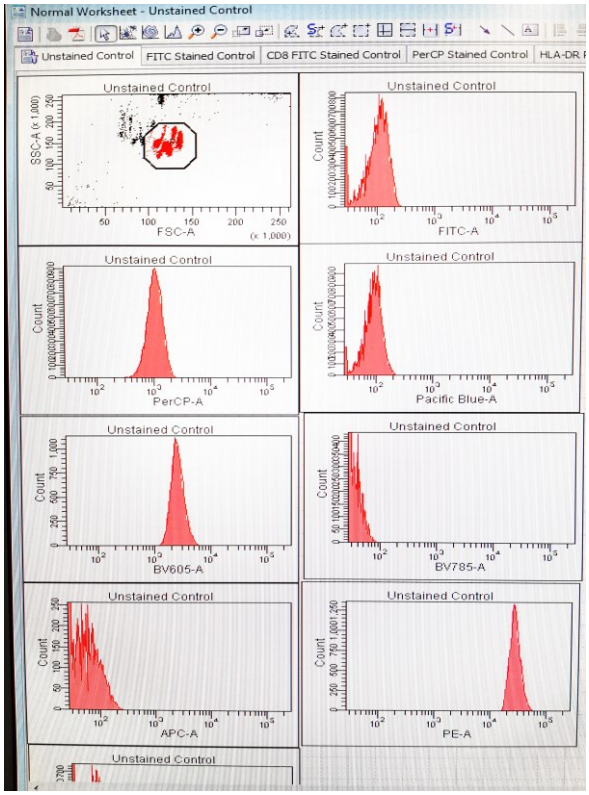


- check your negative bead sample in the “Unstained Control” tube, histograms should look as shown above

  CS&T settings are checked weekly and define the best separation between negative and dim positive populations in a given fluorochrome chanel, therefore it is usually not necessary to change PMT voltages according to your negative beads

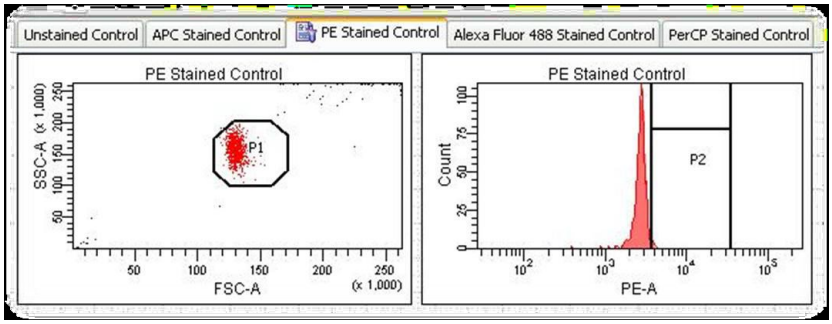
- look at one of your fully stained samples to see if there is any fluorochrome out of range (above 10^5)

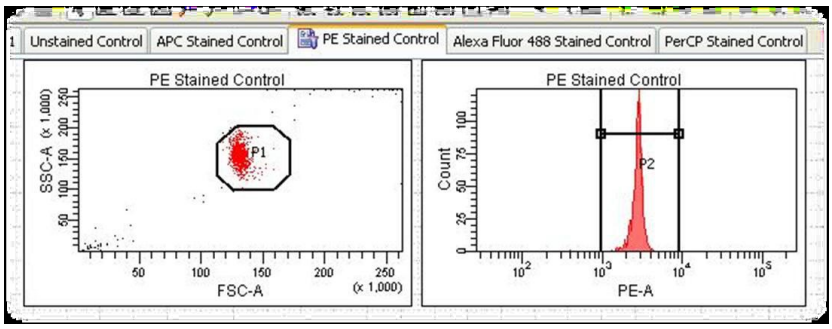
- use your single stained beads in the “unstained control” tube to see if there is any spectral overlap in any other detector



- after checking all single stained beads and setting up the PMT voltages record each single stained control in its specific tube (we recommend 20.000 events, don't use default values)

- adjust gate P2





- you can even set up the P2 Gate smaller to the Population

1.2.2 Setting the spillover values with negative and positive beads or cells in the same tube

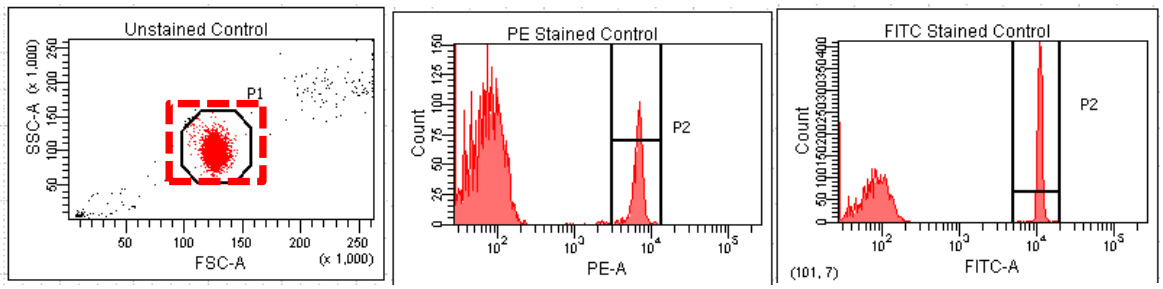
- **if you don´t use a separate negative control, you have to gate also the negative cells or beads in your compensation control tubes**

- mix your stained control and load it to the **Sample Injection Port (SIP)**
  Press **"Low"** and **"Run"**

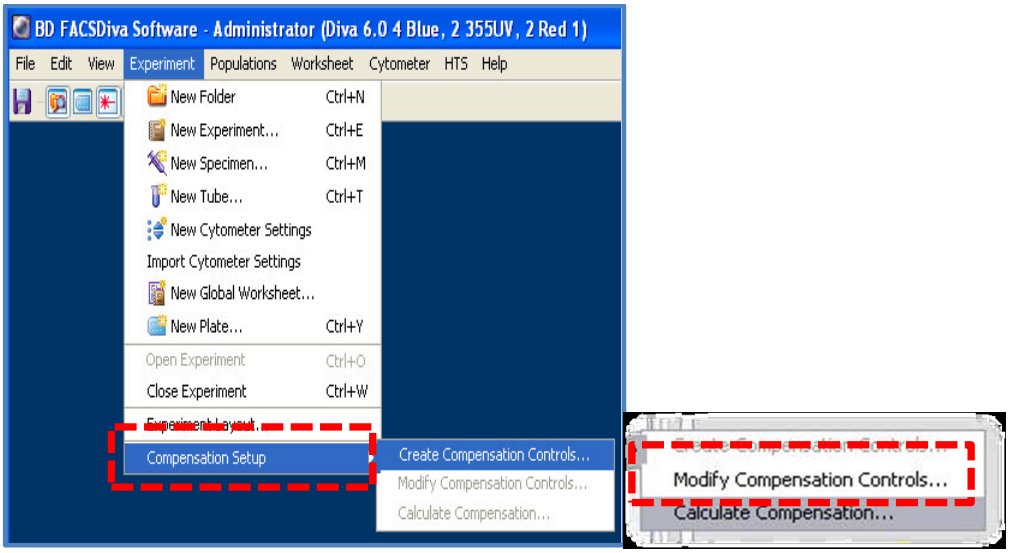
- click **"Acquire Data"** and wait a few seconds until the threshold rate is stable

- adjust gate P1 to include your population of interest

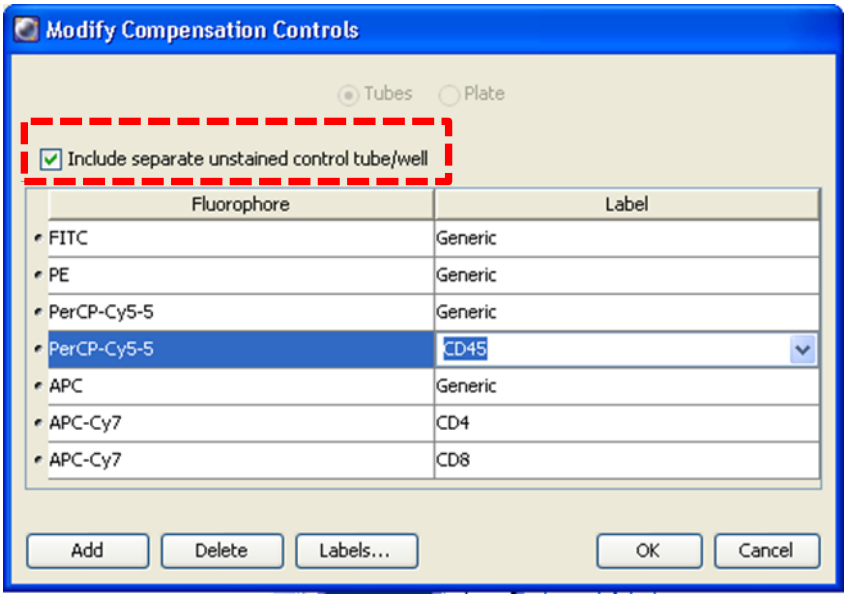
- adjust P1 to all samples, right mouse tab > **"Apply to all Compensation Controls"**



- check negative bead sample in the “Unstained Control” tube, histograms should look as shown above
- look at one of your fully stained samples to see if there is any fluorochrome out of range (above 10^5)
- use your single stained beads in the “unstained control” tube to see if there is any spectral overlap in any other detector
- remove the “unstained control tube/well”
  Experiment > Compensation Setup > Modify Compensation Controls

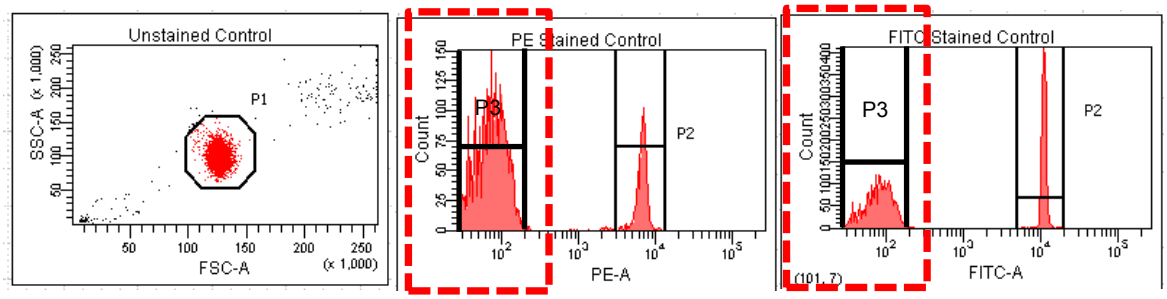


- uncheck **“Include separate unstained control tube/well”** and click **“OK”**



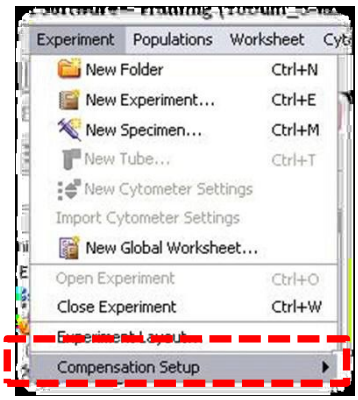
- **record** data for all single stained controls in its specific tube (**we recommend 20.000 events,** don´t use default values)

- **in contrast to section 1.2.1 you have to draw a gate around the negative cells or beads in your compensation control tubes**

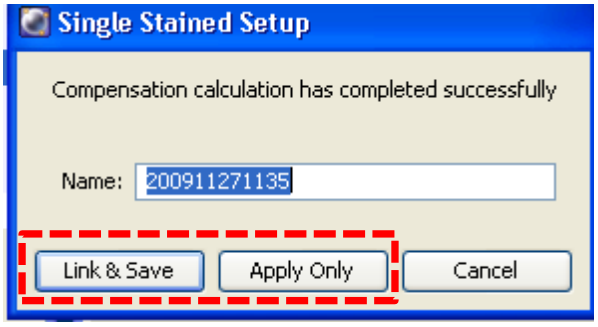


1.3 Calculating Compensation

- click **“Experiment” > “Compensation Setup” > “Calculate Compensation”**



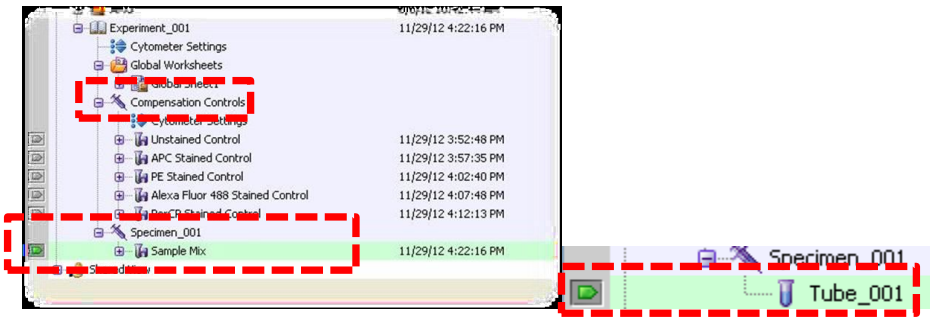
- the software will calculate compensation for all parameters



- **“Link & Save”** the compensation settings will be used in your experiment and saved in the catalog **(“Cytometer” > “Catalog”** [allows to import compensation into other experiments])
- **“Apply Only”** the compensation settings will only be used in your actual experiment

- switch back to **Global Worksheet** by clicking on the top left icon **1.**

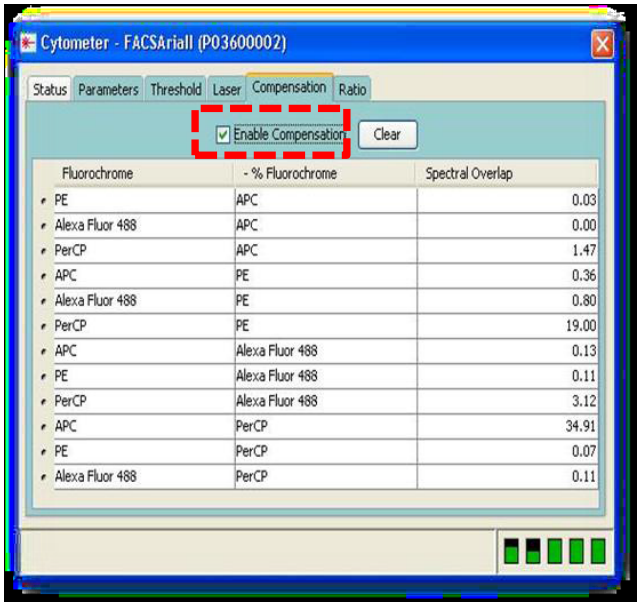
- click on one sample tube
- make sure that the tube pointer on the far left of the sample tube is selected (**green**)



- check if compensation is calculated successfully

  “Cytometer” > **“Compensation”**

- check if **“Enable Compensation”** is marked



- now you are ready to collect data for your samples

1.4 Measure your Samples