

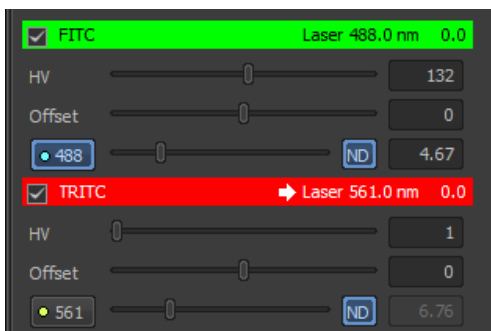
FRET Protocol

When using the Specified Bleedthrough and related methods, a set of four images is required for each sample (substitute GFP and mCherry for any FRET pair):

- Excited with the **GFP** (Donor) exciter and looking in the **GFP** (donor) channel (called **Dd**)
- Excited with the **GFP** (Donor) exciter and looking in the **mCherry** (acceptor) channel (called **Da**)
- Excited with the **mCherry** (Acceptor) exciter and looking in the **GFP** (donor) channel (called **Ad**)
- Excited with the **mCherry** (Acceptor) exciter and looking in the **mCherry** (acceptor) channel (called **Aa**)
- These should be taken for a Donor-only sample, an Acceptor-only sample, and the FRET sample.

To quickly and easily capture the 4 sets of images, create two optical configurations.

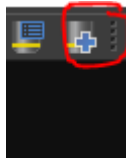
First set the 488 laser power and collect in the 488 channel and 561 channel as set up below.



You can click on the channel name and rename to Dd and Da

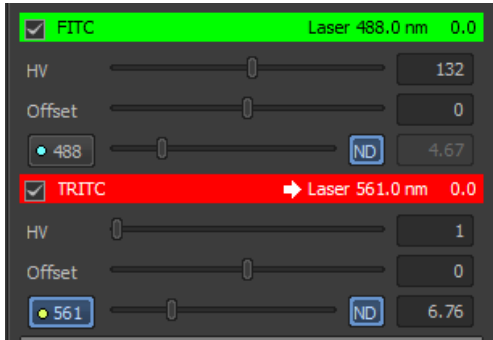


Create a new Optical configuration related to these settings. To do this you click on the “New OC

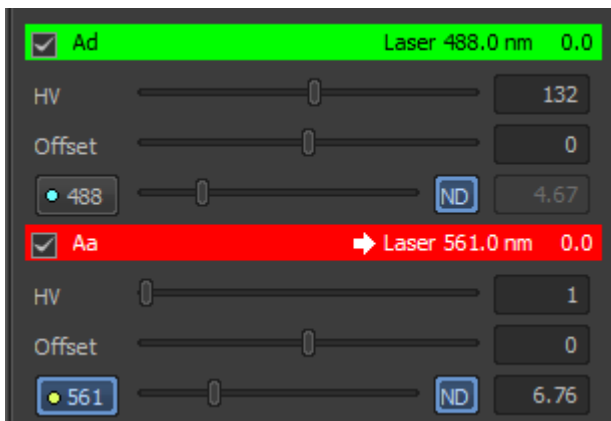


button” once you have it set up as required. Name this OC DdDa

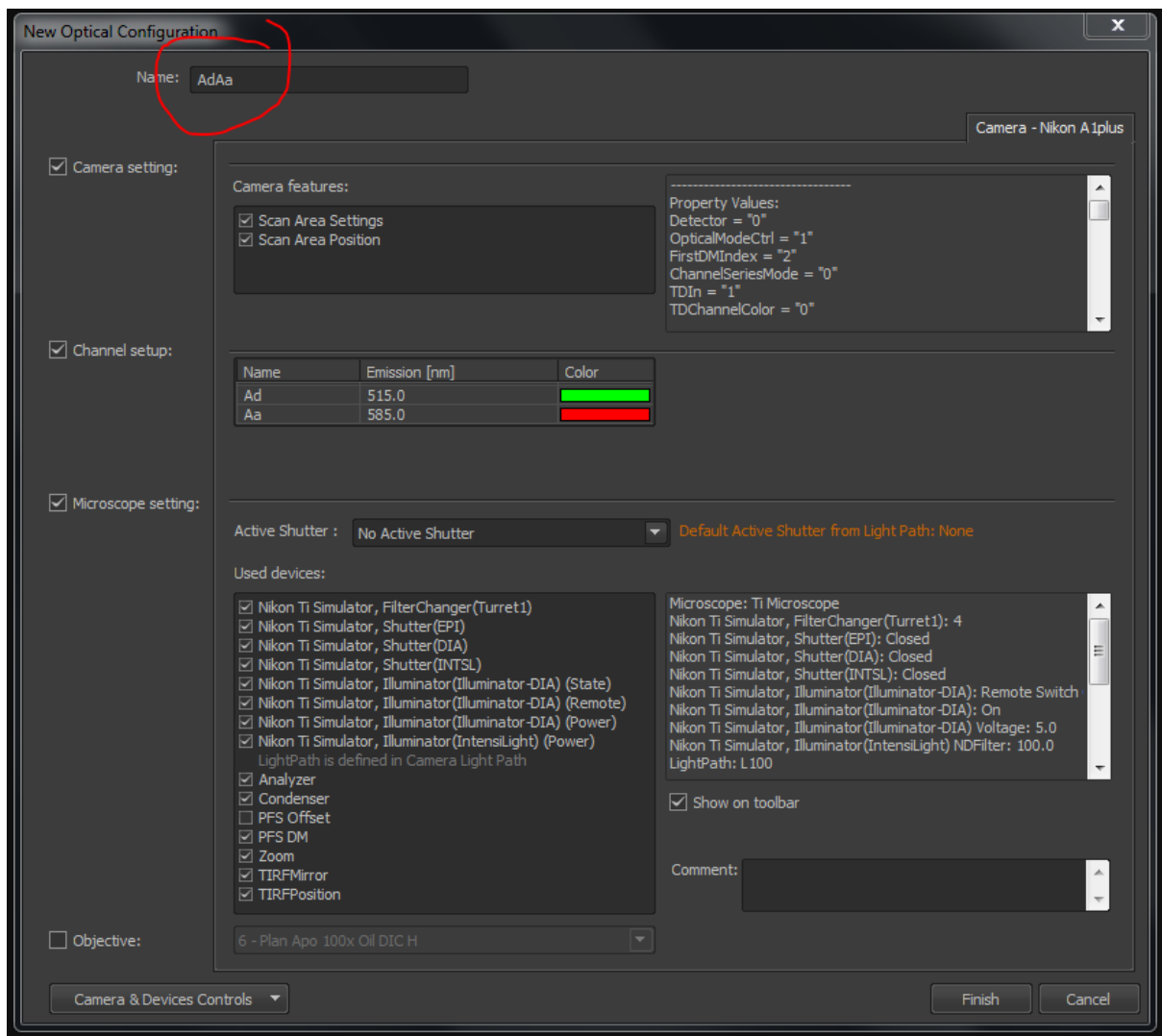
Then set the 561 laser power and collect in 488 and 561 channel as below



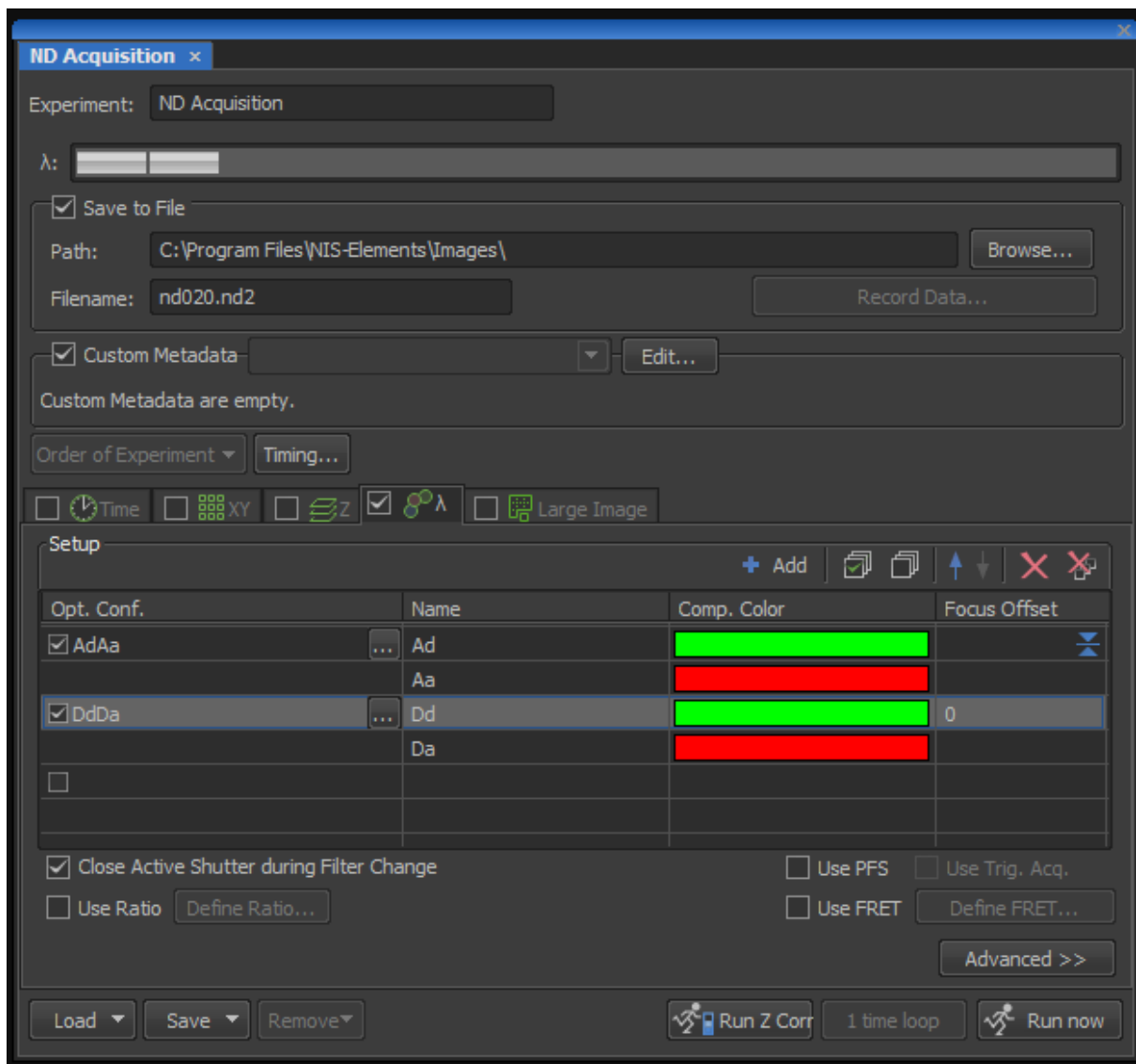
Again rename the channels Ad and Aa



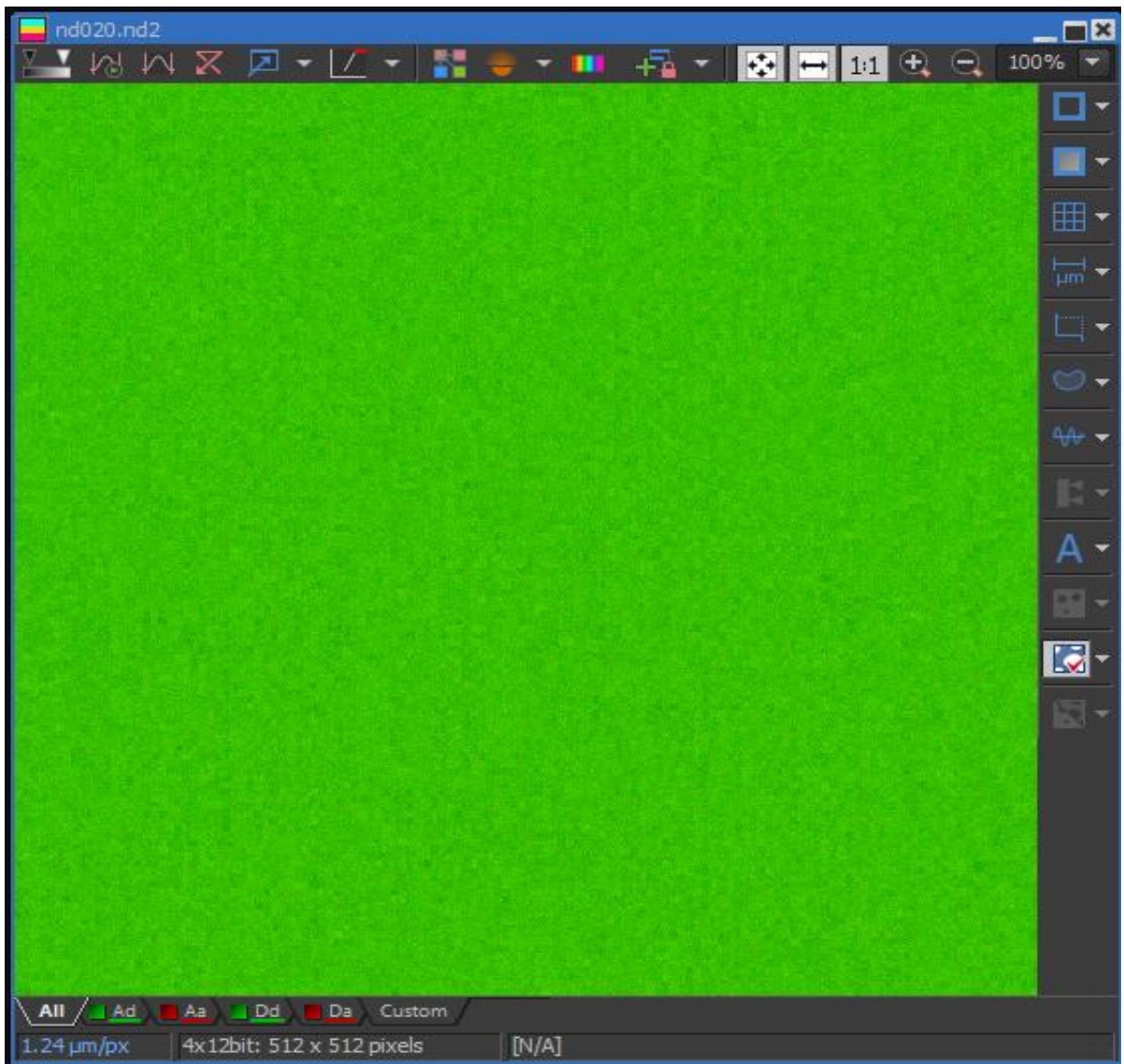
And create an OC relating to these settings named AdAa



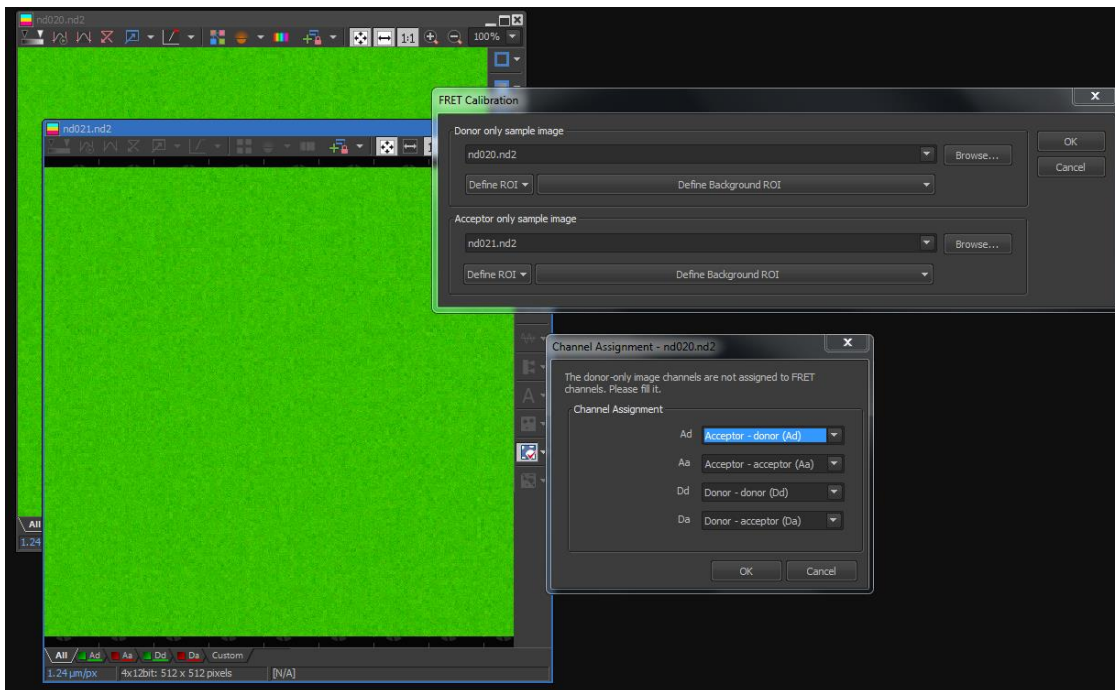
Then image your control samples and main sample by setting up an ND acquisition with the two OCs selected in the wavelength tab



The resulting image will have the 4 tabs along the bottom



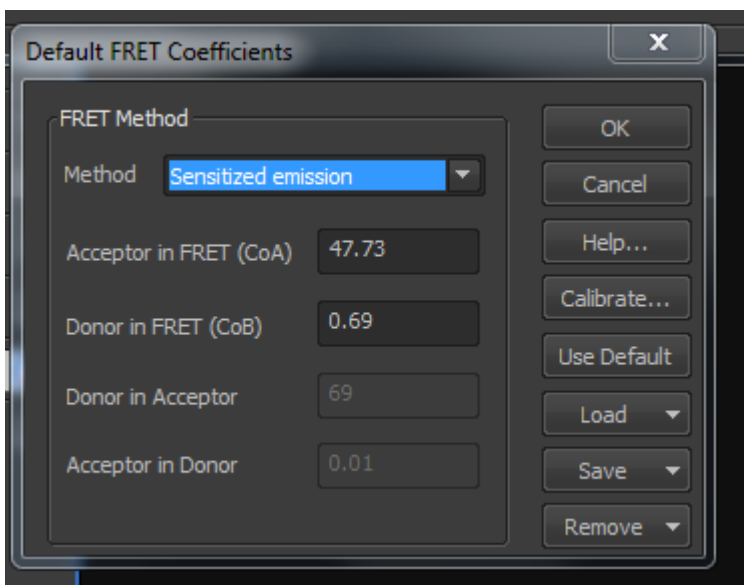
First capture your control images and use these for the calibration.



Capture the an image with donor only and one with acceptor only using the two OCs created previously. Then select Applications> FRET calibration to get the pop up window shown above. With the images open you can select the open files from the drop down menu that relate to the Donor only and acceptor only image. You can draw ROIs in the images to better show the regions of sample too. It is recommended to set a background ROI too.

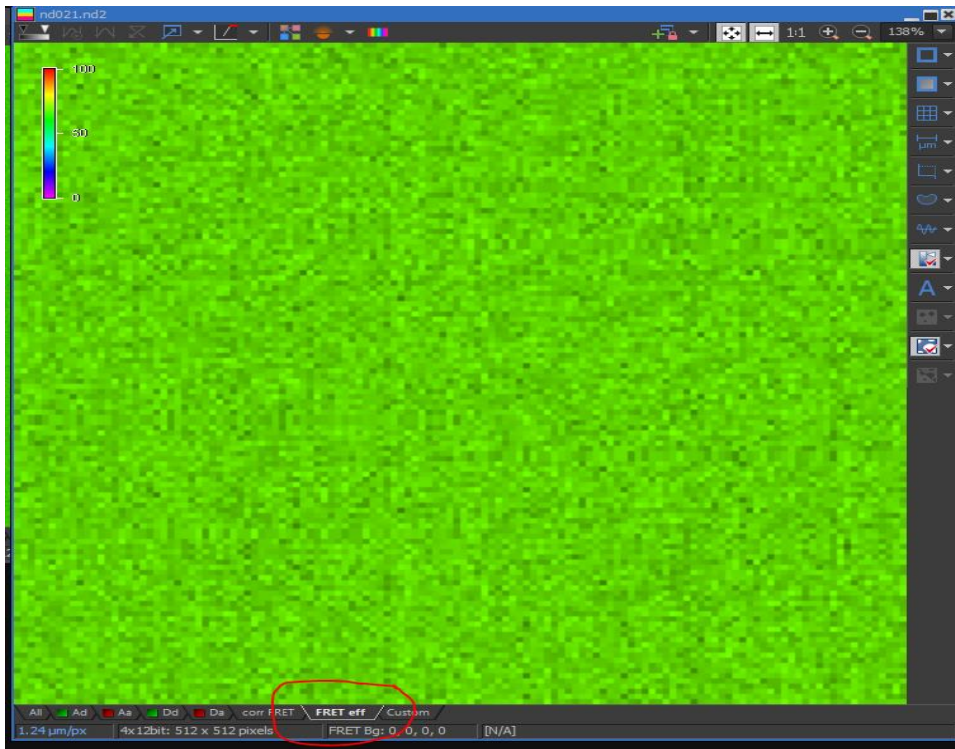
A second window may pop up when you click ok asking to specify the channels Ad, Aa, Da, Dd - this is easily done if you have the OCs set up as described above as the tabs should be automatically correct as they are on the window above.

Finally a FRET coefficients window will appear

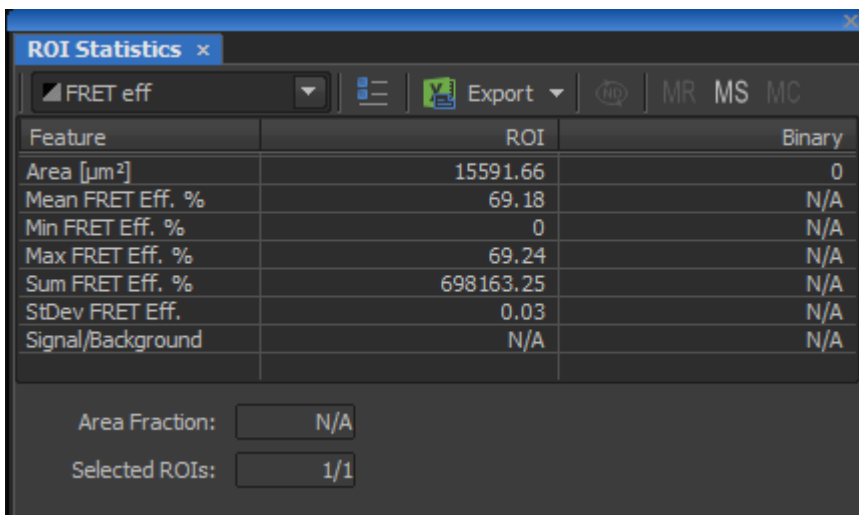


Once you have the calibration, simply collect your sample image again using the two OCs. Then under Applications select FRET view.

This will add a couple more tabs at the bottom of the captured image window – Corrected FRET and FRET Efficiency. Right click in the image window to select show scale which indicates the scale of 0 to 100% FRET (violet to red)



To get detailed values draw an ROI and then right click away from the image and select analysis controls> ROI statistics. Select FRET Eff fro the drop down menu and it will give you details of the values for the ROI



I really hope this all makes sense. Sorry it is a bit long winded. I actually created some OCs ready for you when I was in looking at the system with Leandro but im not sure if they were on a particular log in so you might not see them. Leandro should be able to advise too though if you have any problems.

Please let me know how you get on.