Progenesis SameSpots Tutorial

Including QC, Pre-filtering Stats and Picking

Introduction

This tutorial takes you through the complete analysis of a 4 gel 2D DIGE experiment* (12 images in total) using the unique SameSpots workflow. Specifically you will start with Image QC and the stages involved in experimental setup then image alignment, followed by analysis that creates a list of interesting spots which are explored within Progenesis Stats using multivariate statistical methods then onto spot picking and reporting.

"The statistical analysis of quantitative protein expression data from 2-DE proteomic experiments has previously been compromised by extensive "missing" data, due to problems of spot detection and matching between 2-D gels within an experiment. This problem has been completely overcome with the advent of Progenesis SameSpots, allowing us to apply more rigorous statistical tests. SameSpots provides much faster analysis of large 2-D gel data sets because of the reduced need for user interaction to carry out tasks such as spot editing and manual matching between gels. The results are therefore more objective and we have much greater confidence in their statistical validity."
Mike Dunn, Professor of Biomedical Proteomics, UCD Conway Institute, Ireland and President of the British Society for Proteome Research

How to use this document

You can print this tutorial to help you work hands-on with the software. The complete tutorial takes about 30 minutes and is divided into two sections. This means you can perform the first half focused on image alignment and complete the second half of analysis and exploring interesting results at a convenient time. If you experience any problems or require assistance, please contact us at **download@nonlinear.com**

"Everything about Nonlinear Dynamics makes it a great company to work with. The sales team really knows the product, technical support is excellent and readily available, and the software package itself is superb. The SameSpots upgrade gives an already great software package even greater accuracy. Being able to perfectly line up our gels increases the confidence we have in our results and reduces the chance for false negatives and positives. We would not do 2d gel analysis without it. The whole analysis process is easy, is highly accurate, and provides statistically meaningful results."
**Merry L. Lindsey, Ph.D., Assistant Professor, The University of Texas Health Science Center at San Antonio**

## How can I analyse my own images using SameSpots?

You can freely explore the quality of your images using Image QC and then licence your own images using this evaluation copy of Progenesis SameSpots. Instructions on how to do this are included in a section at the end of the tutorial document. Alternatively if you would like to arrange a demonstration in your own laboratory contact **download@nonlinear.com** and we will help you.

*A 2D DIGE experiment used for demo purposes. SameSpots offers the same benefits for single stain analysis. If you are analysing single stain experiments please contact download@nonlinear.com for more information.

Workflow approach to 2D image analysis

Progenesis SameSpots adopts an intuitive 'Workflow' approach to performing 2D analysis for both 'Single Stain' and 'DIGE' image sets. The following tutorial describes the various stages of this workflow (see below) focusing mainly on the stages from Alignment to Reporting.
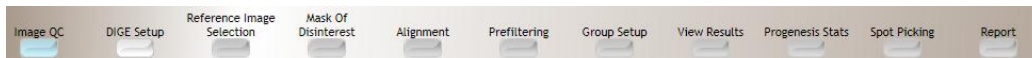
| Stage	Description	Page
Image QC	**Image QC:** Selection, review and manipulation (including Cropping) of Images for analysis.	5-6
DIGE Setup	**DIGE Setup:** Assignment of images to multiplexes (gels)	7
Reference Image Selection	**Reference Image Selection**: Select image to align to.	8
Licensing	**Licensing**: available ONLY for unlicensed images (Appendix 1)	9
Mask Of Disinterest	**Mask of Disinterest**: exclude areas from further analysis	9
Alignment	**Alignment**: automatic and manual image alignment	10-17
Prefiltering	**Prefiltering**: automatic analysis and filtering of spot detection	18-19
Group Setup	**Group Setup**: defining one or more group set ups for analysed aligned images	19-20
View Results	**View Results**: review and validate results, edit spot detection, Tag spot groups of interest and select spots for further analysis	20-28
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Restoring the DIGE SameSpots Tutorial

When you open Progenesis SameSpots click on the 'tutorial experiment' link.
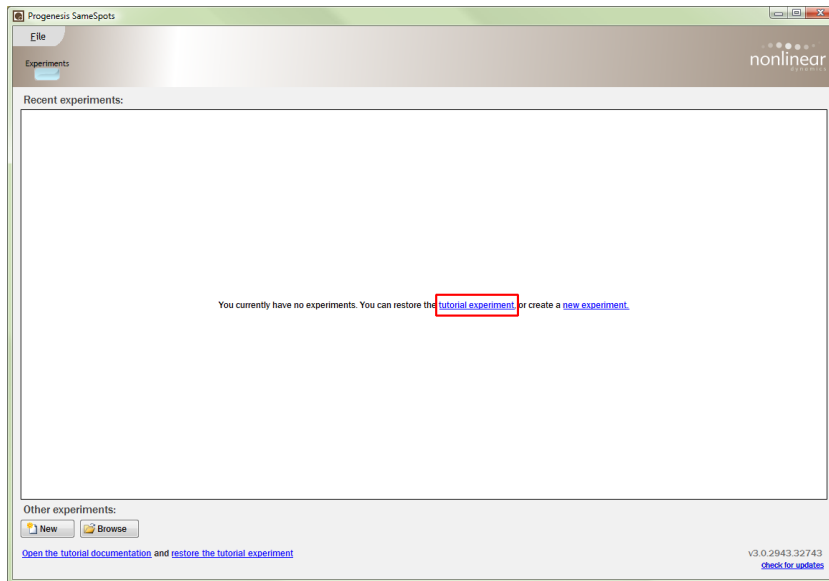
This opens this tutorial document as a pdf and also opens the dialog box enabling you to create a new folder to contain the SameSpots Tutorial.
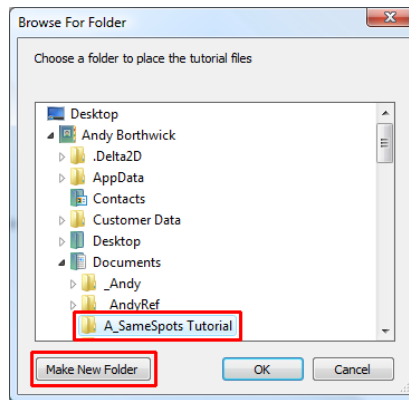
As an example you can create a new folder called 'A_SameSpots Tutorial' in the 'Users' 'Documents' folder. Make sure this folder is current and click OK. A **Restoring Tutorial** progress bar appears and the image files (includes an example image of a Pick Gel) for the SameSpots Tutorial are placed in this new folder.
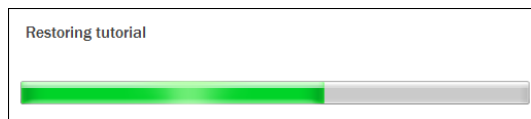
The Experiment Dialog will appear with the details for the SameSpots Tutorial already entered
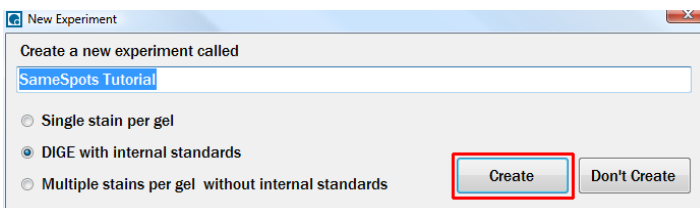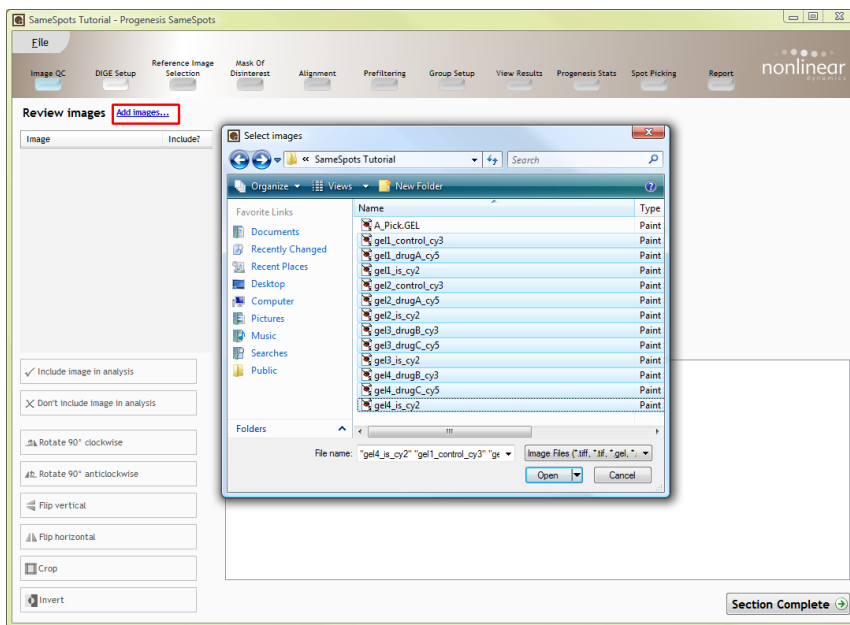

Click Create to open the Image QC stage of the SameSpots Workflow.

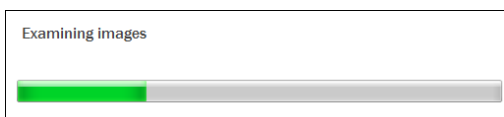## Stage 1: Review and Quality Control (QC) of image set for analysis

The Image QC module will open.

Locate your images using the Add images.. link.

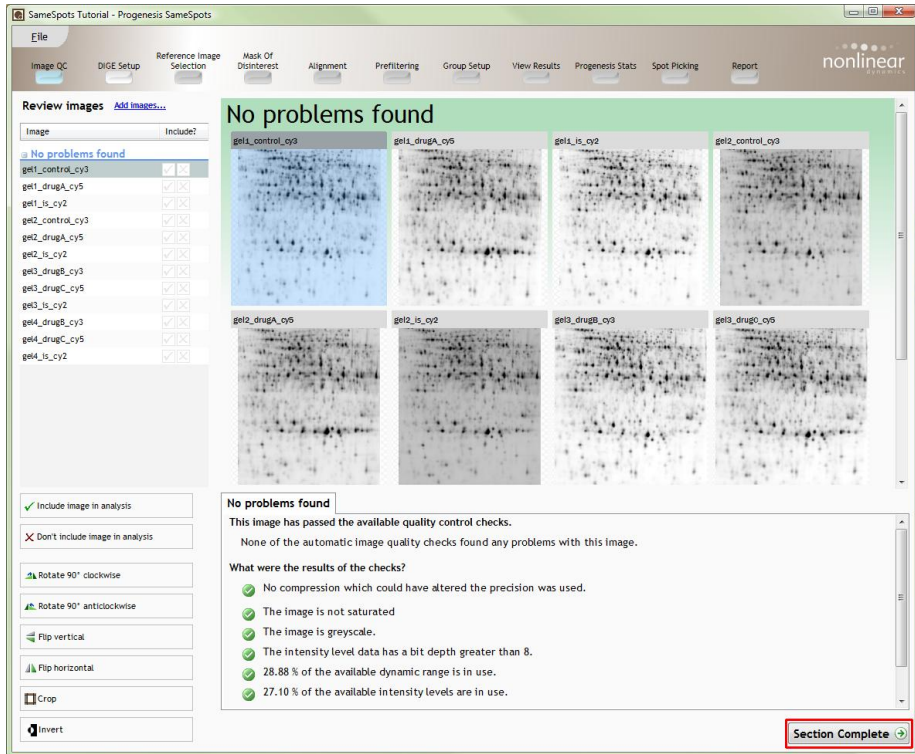Locate and select all the images **except A_Pick.gel** from the folder you have just created.

On loading the selected images your data set will be automatically examined by the Quality control application.

Note: For a large number of images this may take a few minutes.

The Image QC will refresh reporting on your images.

For each image, a dialog outlines the image properties and where they exist, highlights the images with problems. In this case 'No problems found' has been reported for each image with regards to the image capture process.

Note: if you have selected any images for the current analysis that you do **NOT** want to include then select them from the list and click on the **Don't include image in analysis** in the tools or just click on the X box next to the image on the list
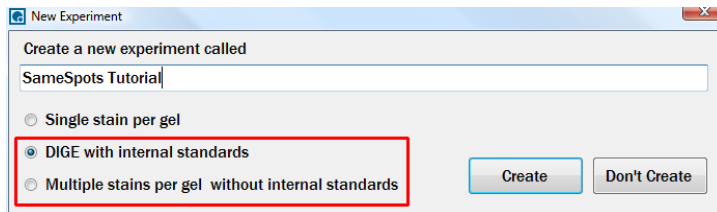

For this Tutorial analysis use all the images

Note: if required you can also crop the images during the workflow

Now move to the next stage in the workflow by clicking **Section Complete.**

Stage 2: DIGE setup

This stage in the analysis workflow opens if you have selected either the 'DIGE' or the 'Multiple Stains' formats when creating a new experiment.


Having selected the **DIGE with internal standards** format this stage allows you to assign the gel images to the correct multiplex (physical gel).

The experiment used in this tutorial explores changes in protein expression between three drug treatments (A, B and C) and a control. As this is a DIGE experiment each gel generates 3 images: a standard and two sample images, the gels were run as shown in the table.

Gel No.	Internal Standard	Sample 1	Sample 2
gel 1	gel1_is_cy2	gel1_control_cy3	gel1_drugA_cy5
gel 2	gel2_is_cy2	gel2_control_cy3	gel2_drugA_cy5
gel 3	gel3_is_cy2	gel3_drugB_cy3	gel3_drugC_cy5
gel 4	gel4_is_cy2	gel4_drugB_cy3	gel4_drugC_cy5

First click on 3 images that have been run on the same gel, then right click and select **Add to new gel…** The images will appear on the panel to the left.
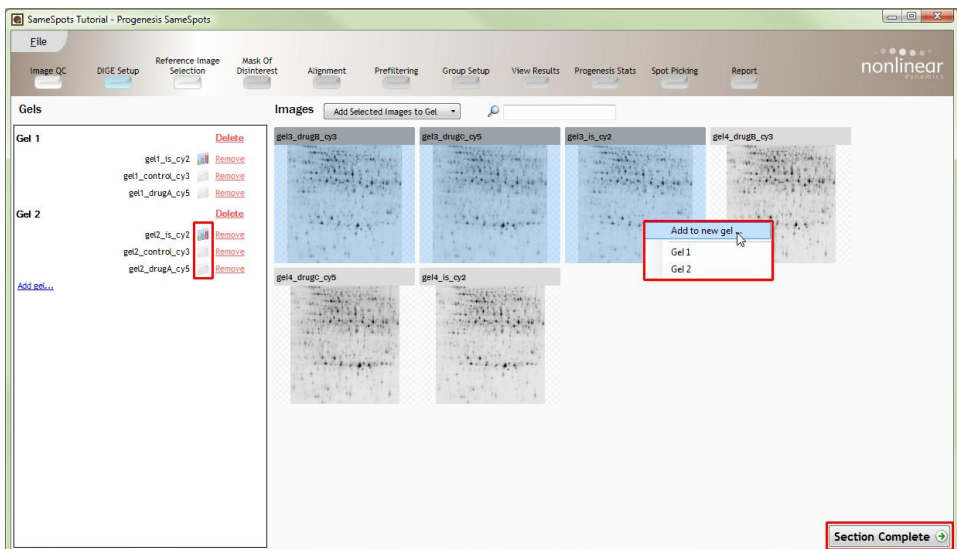
If the gel naming convention is recognised then the internal standard image will be tagged automatically. If not, then click on the icon to the right of the gel name to reassign.
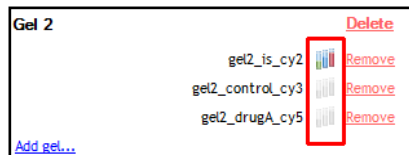
Note: the selected images can also be 'drag 'n' dropped' onto the **Gels** panel to create a new gel or to add them to an existing Gel

For this tutorial there are 4 DIGE gels or multiplexes, so drag all the images into the Gels panel.

Now move to the next stage in the workflow by clicking **Section Complete.**

Stage 3: Reference Image Selection

This stage in the analysis workflow allows you to review and select the most appropriate Reference Image to align all the images to.

In the DIGE format you are only offered the images which are set as Internal Standards, in this example the Cy2 images, where gel1_is_cy2 is used.
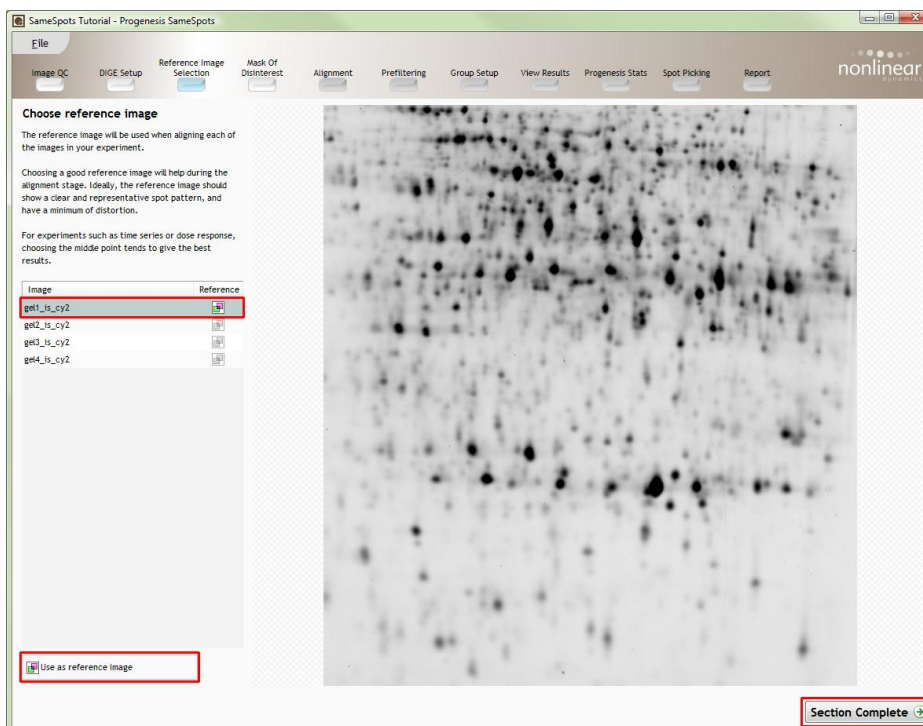
To select a Reference image either click on the image in the list and then click **Use as reference image** or double click on the image in the list.

Now move to the next stage in the workflow by clicking **Section Complete.**

Stage 4: Licensing

This stage in the analysis workflow will **ONLY** appear in the SameSpots workflow if you are using 'Unlicensed' images to evaluate the software.


For details on how to use Licensing go to Appendix 1 (page 43)

Stage 5: Mask of Disinterest

At this stage in the SameSpots workflow 'mask(s)' can be placed on the image to define areas to be excluded from the analysis (no spot detection is carried out in the 'pink' masked area(s)).

For example: using the **Edges** tool (see below) a mask has been 'drawn' out on the image. The pink area indicates the masked areas which are EXCLUDED from the analysis.
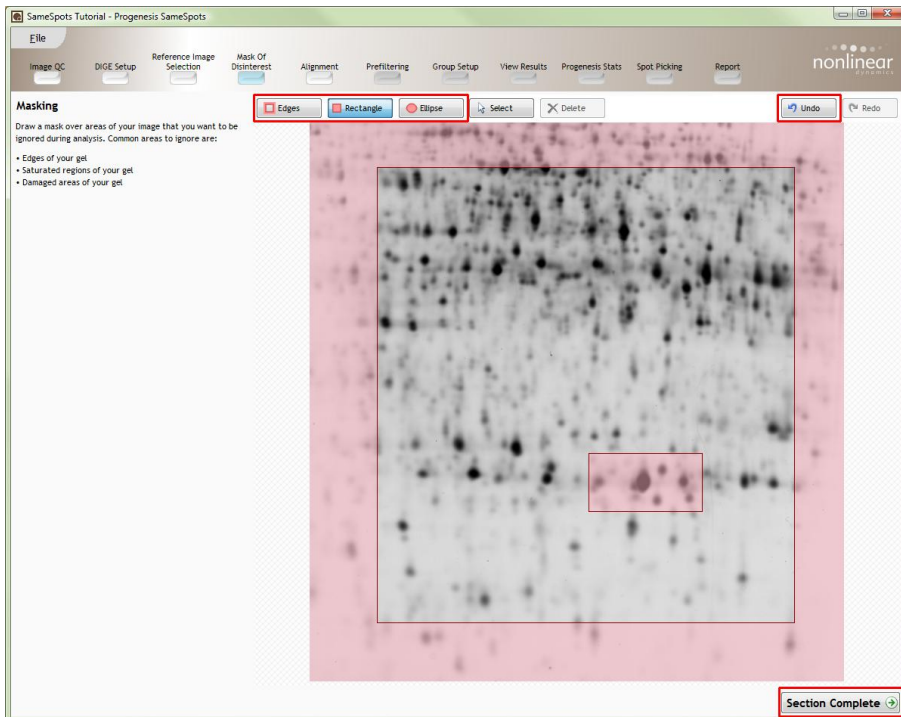
Note: using the **Rectangle and/or Elipse** tools as well, you can mask specific areas of the image which represent over expressed/saturated protein spots

For this tutorial we **DO NOT** require to use a mask so if you have placed a mask use the **Undo** on the top right to remove the mask(s).

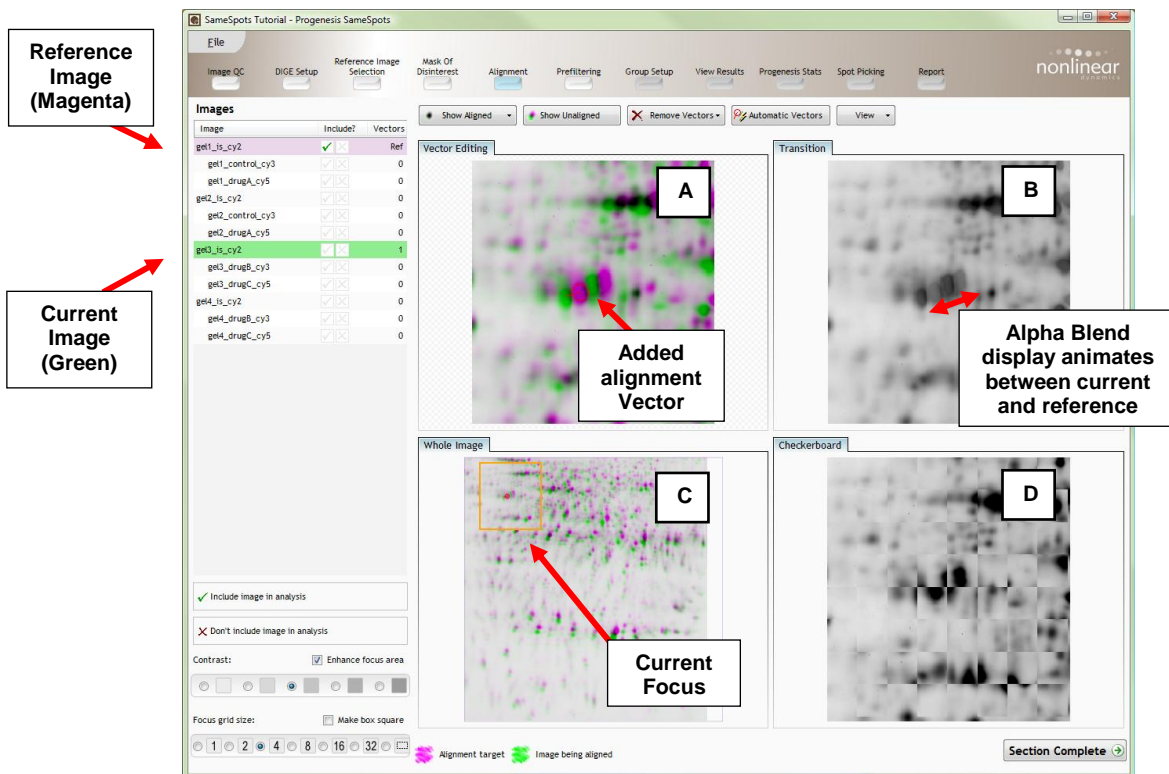Now Click **Section Complete**

Stage 6: Alignment

At this stage Progenesis SameSpots Alignment opens displaying your images

The Program Layout

To familiarize you with Progenesis SameSpots Alignment, this section describes the various graphical features used in the alignment of the images.

To setup the display so that it looks similar to the one below:

- Click on the spots shown in the current focus (orange rectangle) in Window C, this will update windows A,B and D as shown below

- In window A **click and hold** the left mouse button on the smaller of the two main green spots, it should auto align.

- If the green and magenta spot (immediately to the right) have not aligned automatically then **drag** the green spot over the magenta spot and **release** the mouse button

- The image will 'bounce' back and a red vector, starting in the green spot and finishing in the magenta spot as a circle will now appear as shown below in window A

Reference Image (Magenta)

Current Image (Green)

A

B

Added alignment Vector

Alpha Blend display animates between current and reference

C

D

Current Focus

The experiment structure is displayed on the left of the screen in the **Image** panel based on the gel/multiplex information used in the setup described in Stage 2.

Images panel: shows the image that is currently being aligned in green, and the image it is being aligned to in magenta.

The **Ref** gel **(top of image panel)** for any experiment is the image that you chose to align all the images to, in this case gel1_is_cy2 highlighted in magenta and Ref in the Vectors column.

Images		
Image	Include?	Vectors
gel1_is_cy2	✓ ✗	Ref
gel1_control_cy3	✓ ✗	0
gel1_drugA_cy5	✓ ✗	0
gel2_is_cy2	✓ ✗	0
gel2_control_cy3	✓ ✗	0
gel2_drugA_cy5	✓ ✗	0
gel3_is_cy2	✓ ✗	1
gel3_drugB_cy3	✓ ✗	0
gel3_drugC_cy5	✓ ✗	0
gel4_is_cy2	✓ ✗	0
gel4_drugB_cy3	✓ ✗	0
gel4_drugC_cy5	✓ ✗	0

In a DIGE experiment alignment is a two stage process where each Sample image is aligned to its internal standard and each internal standard image is aligned to the overall Reference image.

Vector Editing (Window A): is the main alignment area and shows the current **focus** rectangle as shown in Window C. The current image is displayed in green and the chosen reference image is displayed in magenta. Here is where you place the alignment vectors.

Transition (Window B): uses an **alpha blend** to animate between the current and reference image. Before the images are aligned, the spot appears to move back and forwards. After alignment, they will appear to pulse if correctly aligned. During the process of adding vectors, this view will change to show a zoomed view of the area being aligned to help accurate placement.

Whole Image (Window C): shows the **focus** for the other windows. When you click on the image the orange rectangle will move to the selected area. The focus can be moved systematically across the image using the left and right cursor keys. The focus grid size can also be altered using the controls in the bottom left of the screen.

Checkerboard (Window D): shows a checkerboard view of the current image interleaved with the reference image. When the area is aligned the edges of the squares start to merge.

The unique use of the alpha blend and checkerboard views helps to make highly accurate alignment of spot borders more obvious to the human eye.

## Fully automatic calculation of alignment vectors

A more manual approach to the generation of alignment vectors can be used. This helps when the images you are aligning display 'marked' positional shifts as a result of the capture procedures.

To try the more manual approach move forward to page 13

To use the fully automatic approach first remove the manually added alignment vector (from the previous section) by right clicking on the vector in the **Vector Editing** window.

Note: cropping images to approximately the same visible area in the QC stage can improve the performance of the Automatic Vector Generation.

Then click **Automatic Vectors** on the tool bar
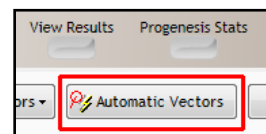
The **Automatic Vector Generation** first displays a list of all the images in the experiment. In this tutorial all the images require the generation of automatic alignment vectors except the chosen Reference Image.
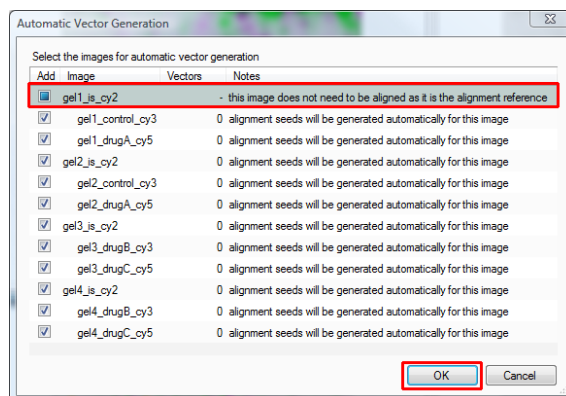
Press **OK** to generate automatic vectors for the two selected image. A progress bar appears.
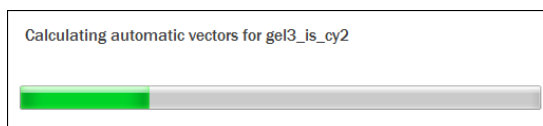
On completion, the number of vectors will be updated on the image list and the vectors will appear (in blue) on the image.

**Now move forward to page 17 to view the alignment vectors that you have generated.**

Manual approach to alignment

In the initial stage of alignment you place vectors between spots on the two images as a starting point for the automatic alignment algorithm. You have already placed an alignment vector on image gel3_is_cy2 in the previous section, to ensure that this image is current:

1. Click on gel3_is_cy2 in the **Image** panel. This internal standard image is to be aligned with the 'RefGel', highlighted in magenta.

2. You only need approximately five **alignment vectors** evenly distributed around the image to give a good result. We will now add **4** additional vectors to this image see below.

3. First ensure that the size of the focus area is set to **4** in the Focus grid size on the bottom left of the screen.

4. Click on spot 2 (see below) in the **Whole Image** window (C) to refocus all the windows. Adjust Contrast as required.
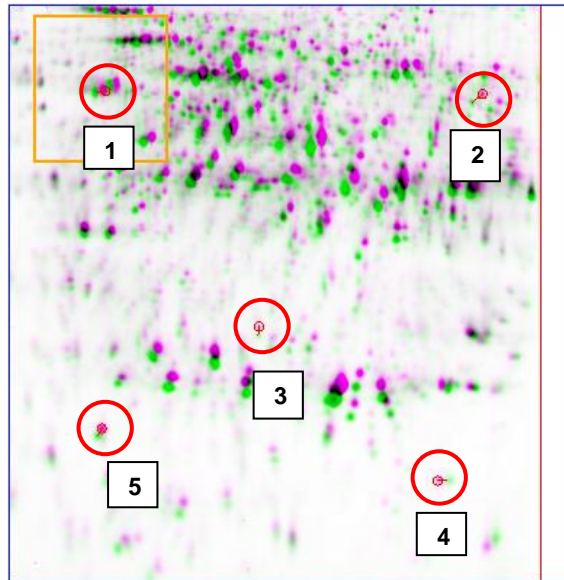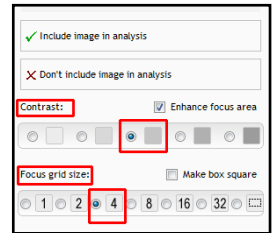
5. Click on the green spot in Window A as shown below. The automatic alignment will place an alignment vector as shown on the next page
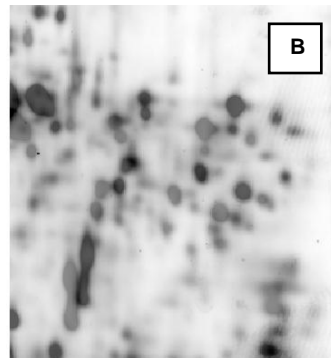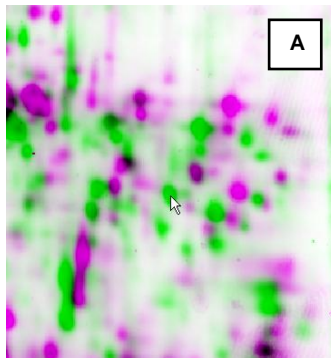
6. If the auto alignment has performed correctly then as you are holding down the left mouse button, the vector will appear as shown below as a red circle with a 'cross hair' indicating that a positional lock has been found for the overlapping spots. **Note**: as you hold down the mouse button, window B zooms in to help with the alignment.
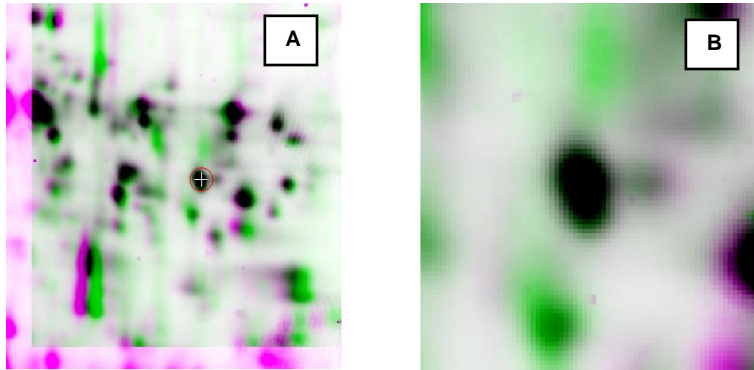
7. On releasing the left mouse button the image will 'bounce' back and a red vector, starting in the green spot and finishing in the magenta spot will appear.
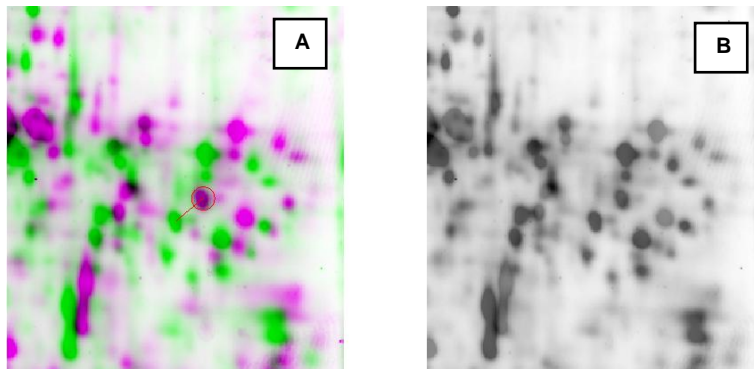
8. If the vector is incorrectly positioned then it may appear as shown below. To remove the incorrect vector, hold the cursor over the vector, and right click to delete the vector.
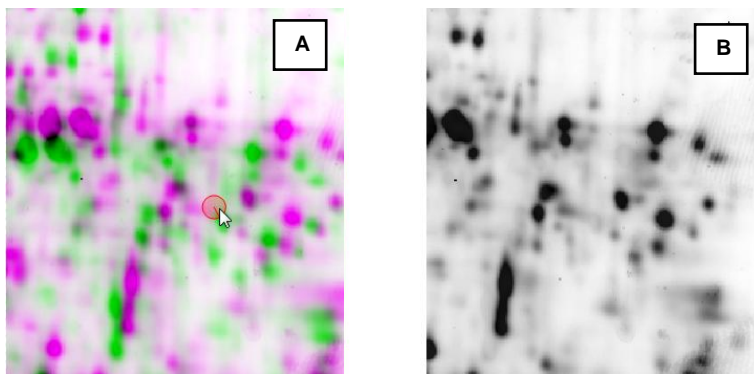
9. This time, in window A **click and hold** the left mouse button on the spot, if the green and magenta spot have not aligned automatically then **drag** the green spot correctly over the magenta spot and **release** the mouse button.

    - Repeat this process for the 3 remaining spots as shown on page 13 so that you have added 5 vectors to **gel3_is_cy2**.

    - Keeping the focus on vector 5, now select gel3_drugB_cy3 from the image list. This sample image will appear aligned with the internal standard image gel3_is_cy2 which is now highlighted in magenta

    - Although this image will appear near perfectly aligned add one vector to a spot in the current focus, this will improve the alignment in the low molecular weight region of the image.

Repeat this process for all the images to be aligned.

Automatic alignment vector calculation using manual vectors

Now click **Automatic vectors** on the tool bar

The **Automatic Vector Generation** first displays a list of all the images in the experiment. In this example, the images you have edited will have their **user vectors** listed.
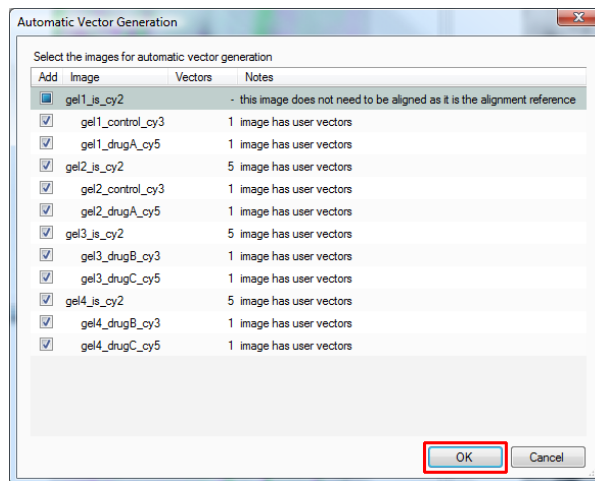
Press **OK** to generate automatic alignment vectors for the images.

On completion, the number of vectors will be updated on the **Images** panel and the vectors will appear (in blue) on the image.

When Progenesis SameSpots Alignment updates click on gel3_is_cy2 on the **Images** Panel and click **Show Aligned** to set the display as shown below.
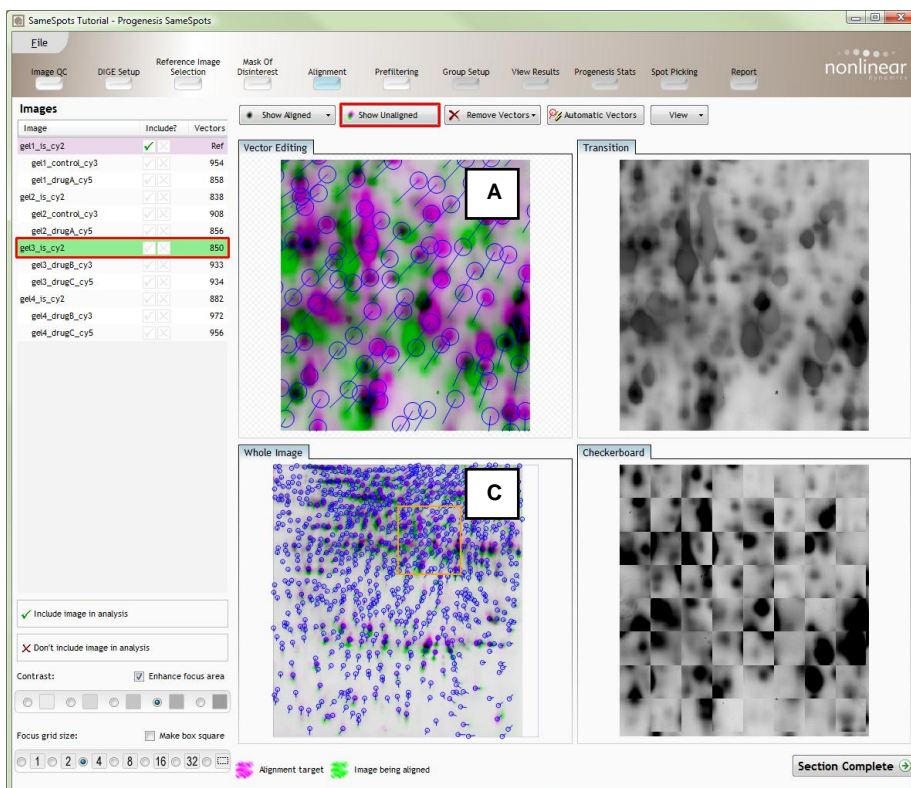

This will show the selected image (green) aligned with the reference image (magenta and showing **Ref** in the vectors column), using all the windows you can see how well the alignment has worked.

The automatically added vectors are shown in blue and if the manual approach was used the manually added vectors appear in red (not shown).

If the alignment has worked well then in Windows A and C the grid lines should show minimal distortion, Window B will show spots pulsing slightly but with no movement of the spots. In the checkerboard view, window D, the grid lines should have faded and images will have merged.

Review vectors in unaligned state

To show the full effect of the manual and automatically generated vectors we can view the unaligned images by pressing **Show Unaligned** on the tool bar.
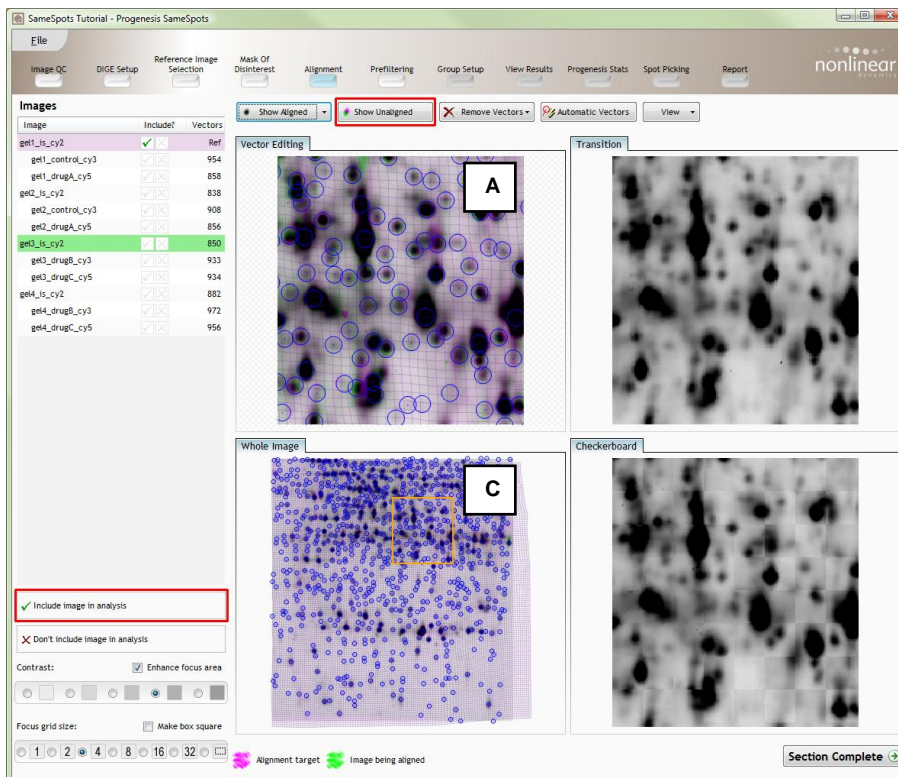
This view helps you to see how well the images will be aligned. The flow of the vectors in Windows A and C indicates how the images will be aligned and also if the vectors flow in the same general direction then the alignment will work well.

To restore the aligned view click **Show Aligned** on the tool bar.

At this point, you should check the automatically placed (blue) vectors. This will be easier with a larger grid size. Make sure the grid size is set to 4 using the **'Focus grid size'** control at the bottom left of the window. Work from top down and advance the focus box using the arrow keys on the keyboard.

In each square, you can, if required edit the vectors to improve the image alignment. In this example, whether you placed the 5 manual vectors similar to the suggested positions or opted for fully automatic approach the images should be aligned well. If required, see **Appendix 2** for hints on editing.

To indicate that alignment of **gel3_is_cy2** is complete and ready for SameSpots Analysis click **Include image in analysis** located underneath the image list. Doing this places a tick against the current image on the Image list. Repeat for all the other images when you have completed review of this image**.**

Stage 7: Prefiltering

Now that you have reviewed your aligned images, you are now ready to analyse them with SameSpots. Move to the next stage, Prefiltering, by either clicking on **Section Complete** (bottom right) or on Prefiltering on the workflow.
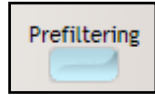
During the few minutes that the automatic analysis requires, a progress bar will appear telling you first that it is applying alignment to the images and then secondly that it is Analysing.
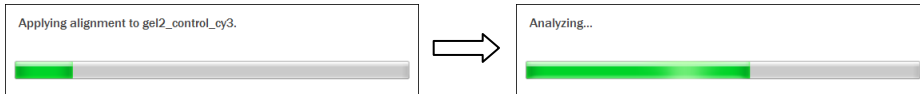

On completion of analysis the Prefiltering page will open displaying the spot detection. If required you can remove spots based on position, area, normalised volume and combinations of these spot properties.

To delete spots at the bottom of the image drag out an area as shown. All spots within and touching the mask will be selected, 67 spots in total as displayed to the left.
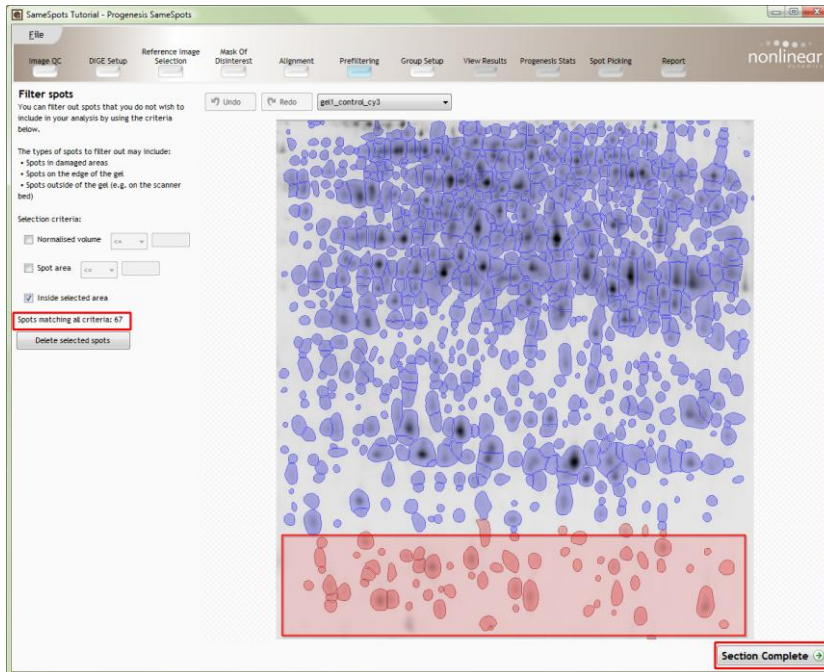
To change the selection criteria for the selected area try left clicking on a small spot and select **Use this spot's area** (see below)
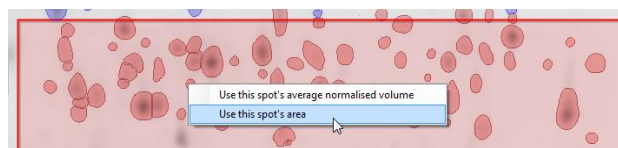
This will reduce the total selected spots in the area to 16 (see below) based on the new selection criteria shown to the left of the image
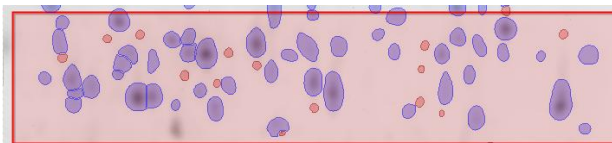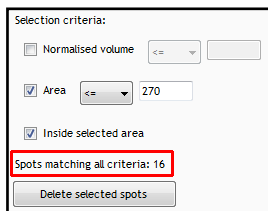
For this tutorial we **DO NOT** need to prefilter the spot detection so undo any filtering you have performed by using clicking **Undo**, located above the image.

Note: if the filter has not been applied it can be removed by un-ticking the boxes in the selection criteria (see above).

To move to the next stage in the workflow click **Section Complete.**

Stage 8: Group Setup for Analysed Images

At this stage in the workflow you can setup one or more groupings of your data.

For this example, group the analysed images to reflect the Biological groupings in the original study. This tutorial contains 4 groups: Control, Drug A, Drug B and Drug C.
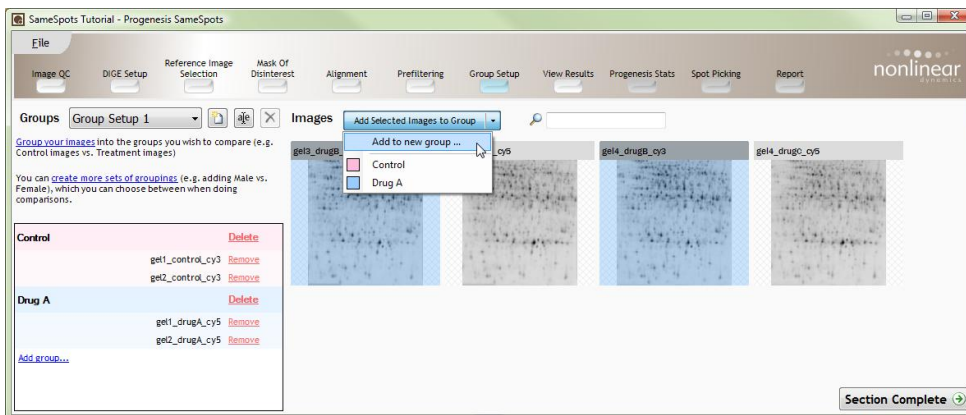
Creating a group

1. Select the images in a group (based on names at top of image) by clicking on the required images (Highlighted as above)

2. Press the 'black triangle' next to the **Add Selected Images to Group** button on the main toolbar.

3. Select **Add to new group…** from the **drop down menu**.

4. A new group will appear in the **Groups** pane on the left panel

5. Rename the group by over typing the new group name (e.g. Drug B)

6. Repeat steps 1 to 5 until all the images are grouped.

To create another Group Setup, for example comparing only the Control with Drug A groups, click on Create a new group setup (see right).
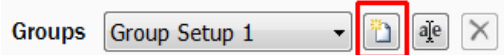
Select **Blank Group** give it a new name (Group Setup 2). The images will reappear in the main window. Create the new groups as described on the previous page.
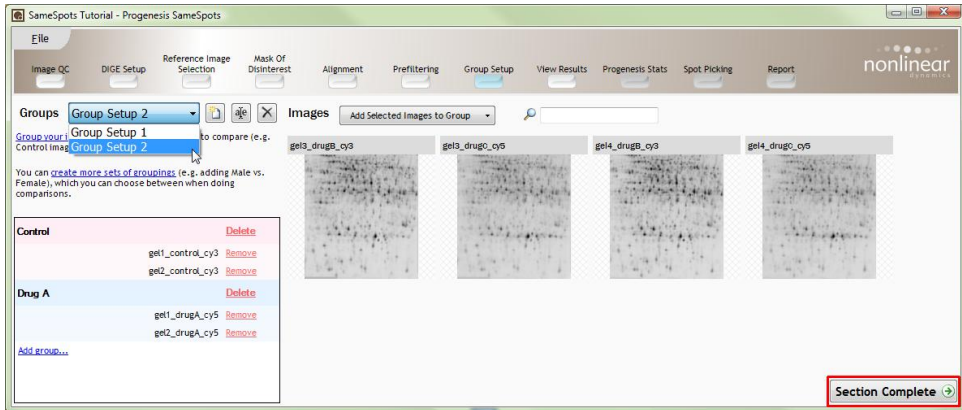
Note: the **Group set up** drop down will now contain both set ups and the ungrouped images for the new setup will remain in the main window.

To move to the next stage in the workflow click **Section Complete.**

Stage 9: Validation, review and editing of results

The purpose of this stage in the Workflow is to review the statistically ranked list of spots, for the current Group Setup (Group Setup 1 is selected here) using the visual tools provided and edit spots as required.

Window A: is a ranked view of the spots based on the p value for the one way **Anova** analysis of the specified sample groups, then by maximum **Fold** change, based on the spots normalised volume, across all the groups being compared. For the current example the highlighted spot (1, as shown above) shows the greatest fold difference between the Control and Drug A groups.

To include this spot in the selection for the next section of the analysis, click on the **Include spot in results** button at the bottom of the table. On clicking the button it will move on to the next spot on the list.

To select a group of spots drag out a selection on the table and click on the **Include spots in results** button (see right)

Review Spots

Grouping: Group Setup 1

Rank	Anova (p)	Fold	Tag ▾	Notes
1	0.000536	16	✓ ✗	📁 ID: 0750
2	0.00638	12	✓ ✗	📁 ID: 0443
3	0.00148	10	✓ ✗	📁 ID: 0760
4	0.000483	9.8	✓ ✗	📁 ID: 0733
5	0.00011	9.8	✓ ✗	📁 ID: 0748
6	0.00102	9.1	✓ ✗	📁 ID: 0706
7	0.000895	6.4	✓ ✗	📁 ID: 0367
8	0.0107	5.9	✓ ✗	📁 ID: 0567
9	0.000644	5.6	✓ ✗	📁 ID: 0589
10	0.00413	5.3	✓ ✗	📁 ID: 0727
11	0.00356	4.6	✓ ✗	📁 ID: 0737
12	0.00207	4.6	✓ ✗	📁 ID: 0859
13	0.000148	4.1	✓ ✗	📁 ID: 0759
14	5.61E-05	4	✓ ✗	📁 ID: 0747
15	0.00203	4	✓ ✗	📁 ID: 0221
16	0.00118	4	✓ ✗	📁 ID: 0582
17	8.94E-05	3.9	✓ ✗	📁 ID: 0743
18	0.00124	3.5	✓ ✗	📁 ID: 0503

| ✓ Include spot in results | 39 marked |
| ✗ Don't include spot in results | 11 marked |

Window B: displays either a montage view of the images focused on the current spot in either 2D or 3D or a Full Image view displaying highlighted spots. When showing 3D the number of images is controlled using the Select Images… link and the orientation and size is controlled by holding down either the left or right mouse button and dragging the mouse accordingly on the window.
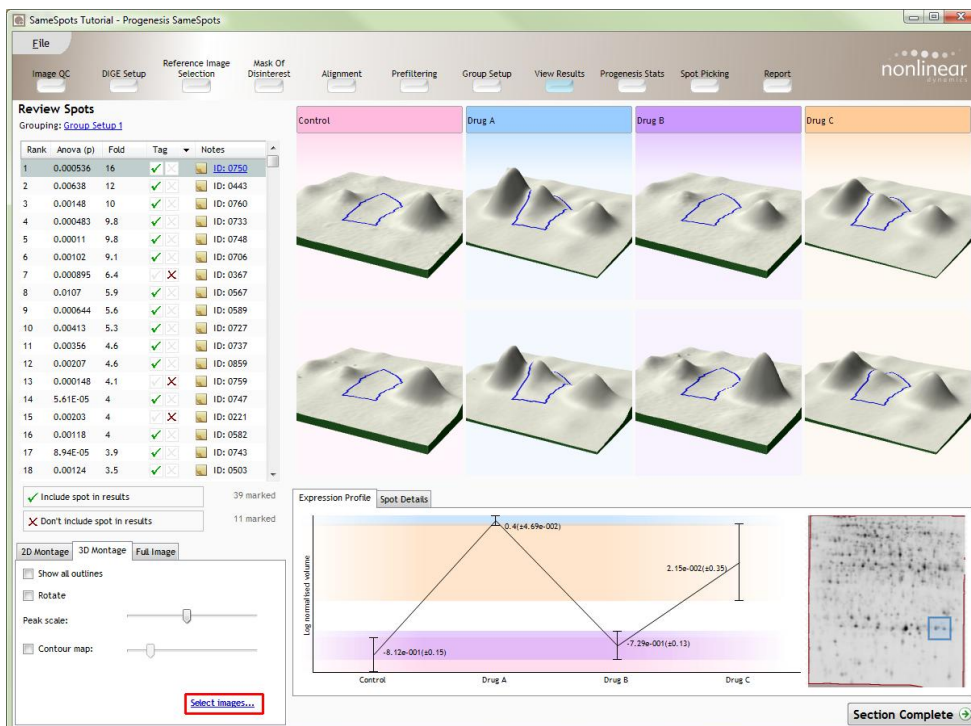
Window C: the **Display** tabs control which view appears in the montage (Window B) and allows you to change the contrast, size and appearance of the montage. To see all the spot outlines tick the **show all outlines** on the 3D tab above.

The Full image allows you to view the location of all the highlighted spots in the Review table. The spot ID and any annotations in the **Notes** field can also be displayed when **Show notes field** is ticked.
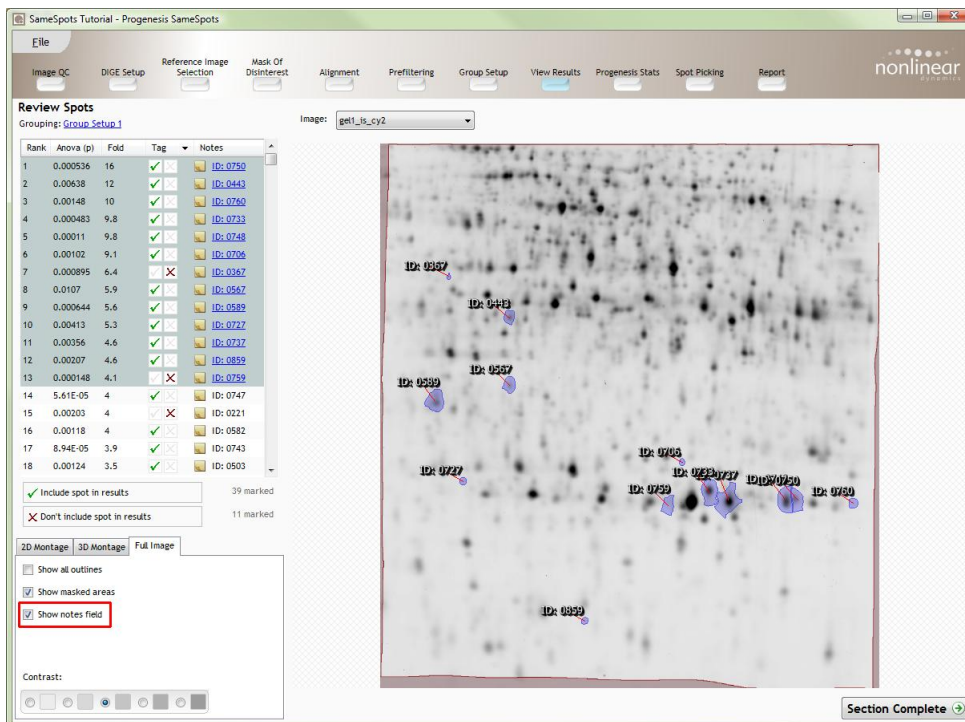

Window D: shows the Expression Profile and Spot Details for the current spot based on the average normalised volume for the groups. The error is shown as +/- 3 standard deviations.

Window E: shows where the current spot is on the image. To change the current location, hold down the left mouse button in the blue square and drag it to a new location. A zoomed view will appear with crosshairs shown in red allowing accurate positioning.

Note: doing this updates the focus of all the other windows.

Also note the grey area outside the red lines on the image this indicates the 'automatic area of disinterest' which can be manually set before Alignment of the images.
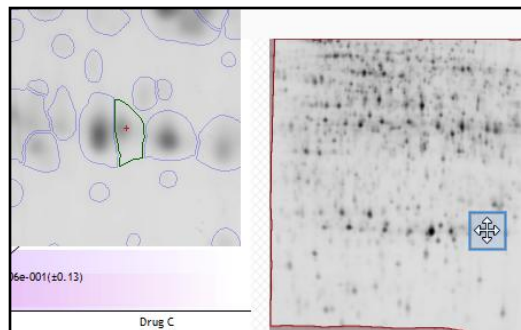
Editing of spots in the View Results stage

As an example of using the editing tools, located at the top of the montage in 2D display mode, we will split the spot currently ranked at 9 in the **Review Spots** list, (actual ranking may vary depending on manual or automatic alignment vector generation).

Splitting a spot

1. Select spot 9 from the **Review Spots** table (Highlighted as above)

2. Select the **split** tool from the edit tools (see above).

3. Click on the centre of one of the spots in the first image of the Drug A group, the cursor will change as shown below, then release and move to reposition the split axis.
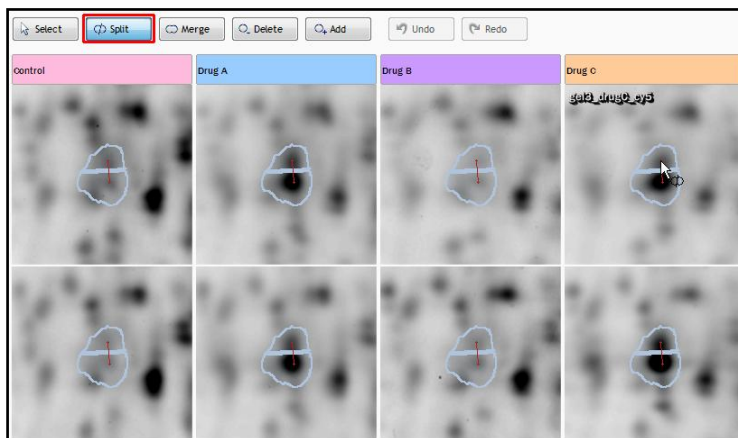
4. When satisfied with the positioning of the axis of splitting, click a second time. The spot will split and the table will display the new values for each new spot. Note: n/a appears in red in the rank column against each half of the split spot.

5. To re-rank the new spots click on **recalculate ranking** at top of the table.

6. If you are not satisfied with the splitting axis use the undo function and retry.

Note: in this example the re-ranked spots have appeared at positions 7 and 13 in the list and are currently unselected.

The other tools: merge, delete and add behave in a similar fashion and their use can be combined to achieve the desired editing of the selected spot.

5	0.000109	9.8	✓	✕		ID: 0748
6	0.00102	9.1	✓	✕		ID: 0706
7	0.000783	6.5	✓	✕		eID: 0002
8	0.000892	6.4	✓	✕		ID: 0367
9	0.0107	5.9	✓	✕		ID: 0567
10	0.00412	5.3	✓	✕		ID: 0727

Note: in the **Notes** field of the Ranked table the 'Edited' spots ID has been renumbered and an 'e' signifying that this spots is a result of an edit has been added. You can edit this as required in the **Notes** field.

Selecting and tagging spots for Progenesis Stats

There are a number of ways to 'refine' your 'Ranked List' of analysed spots before examining them with the Statistical tools in Progenesis Stats. These make use of simple 'Selection' and 'Tagging' tools that can be applied to the various Groupings created in Stage 8 (p 19). Three examples are described below.

Note: the same population of spots is available to all groupings you have created. In this example we have two Group Setups (1 and 2) each applied to the same 890 spots.

1. The simplest approach, making the least assumptions, is to **select** all 890 spots and take them forward into Progenesis Stats.

Review Spots
Grouping: Group Setup 1

Rank	Anova (p)	Fold	Tag ▾	Notes
1	0.000534	16	✓ ✕	ID: 0750
2	0.00637	12	✓ ✕	ID: 0443
3	0.00148	10	✓ ✕	ID: 0760
4	0.000481	9.8	✓ ✕	ID: 0733
5	0.000109	9.8	✓ ✕	ID: 0748
6	0.00102	9.1	✓ ✕	ID: 0706
7	0.000752	6.5	✓ ✕	eID: 0001
8	0.000892	6.4	✓ ✕	ID: 0367

✓ Include spot in results	890 marked
✕ Don't include spot in results	0 marked

2. Alternatively select a group of spots based on simple statistical thresholds; such as all the spots with an Anova p value<0.05 and displaying a 2 fold or greater increase in expression and take these selected spots into Progenesis Stats.

    Note: in this option only 64 spots are taken forward to Progenesis Stats.

Review Spots
Grouping: Group Setup 1

Rank	Anova (p)	Fold	Tag ▾	Notes
61	0.000222	2	✓ ✕	ID: 0610
62	0.00677	2	✓ ✕	ID: 0783
63	0.0167	2	✓ ✕	ID: 0646
64	9.23E-06	2	✓ ✕	ID: 0529
65	0.041	1.9	✕	ID: 0323
66	0.0121	1.9	✕	ID: 0631
67	0.00844	1.9	✕	ID: 0481
68	0.00567	1.9	✕	ID: 0753

✓ Include spot in results	64 marked
✕ Don't include spot in results	826 marked

3. Or use a **combined approach**; incorporating 'Tagging' of interesting groups of spots for one or more **Group set ups** combined with the selection of all 890 spots to take them forward to Progenesis Stats. Then make use of the 'Tags' to control the spots of interest.

Review Spots
Grouping: Group Setup 2

Rank	Anova (p)	Fold	Tag ▾	Notes
1	0.00186	16	✓ ✕	ID: 0750
2	0.00208	9.8	✓ ✕	ID: 0748
3	0.00731	9.6	✓ ✕	ID: 0733
4	0.0126	9.1	✓ ✕	ID: 0706
5	0.00153	9	✓ ✕	ID: 0443
6	0.00463	6.7	✓ ✕	ID: 0760
7	0.00897	6.4	✓ ✕	ID: 0367
8	0.00594	4.7	✓ ✕	ID: 0727

✓ Include spot in results	890 marked
✕ Don't include spot in results	0 marked

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Setting multiple tags for ranked data

To set multiple Tags as shown in example 3 above, first make sure that the Grouping is set to **Group Setup 1** and the table is ordered on Rank (as shown to the right).

Note: the setting of Tags is not affected by the spots 'selection' status in the table.

Now highlight the top 50 ranked spots for Group Set up 1, right click on the highlighted spots and click New Tag.

Give the Tag a name. As the Group setup 1 corresponds to a grouping incorporating all 4 groups in the experiment then; **'Top 50 ranked for All groups'**.

On clicking **OK** the Tag is applied to the spots highlighted in the table.

Now change the Grouping to **Group Setup 2.** Then highlight the top 50 spots in the ranked list. Scroll to spot 20, note that there are a number of untagged spots in the highlighted list.

Right click on the highlighted spots and give the new Tag a name.

As the Group setup 2 corresponds to a grouping comparing the Control and Drug A groups in the experiment then; **'Top 50 ranked Control vs Drug A'**.
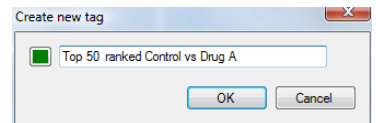
On clicking **OK** the second Tag is applied to the spots highlighted in the table.

Return to Group Setup 1 and create a third Tag for the last 50 spots on the ranked list calling the Tag **Bottom 50 ranked in All groups,** by scrolling to the bottom of the list and highlighting the bottom 50 and creating another Tag in the same way as described above.

To view the Tags and also control the number of spots displayed in the table, click on the drop down selection on the right of the Tag column header. By ticking one or more Tags you can control the number of spots displayed.
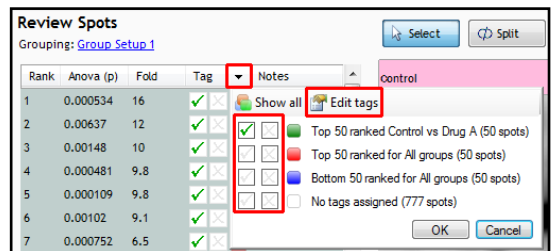
To delete and/or create additional Tags click on **Edit tags** and delete Tags as required.
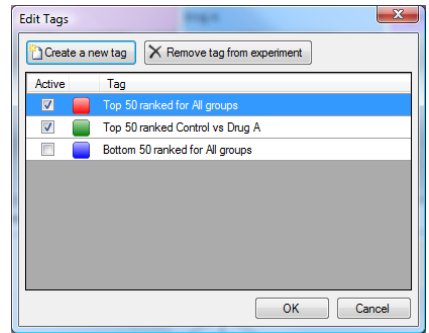
Finally before moving on to Progenesis Stats make sure that **no** Tags are Ticked in the drop down (see above) and also that all 890 spots in the Review Spots table are ticked **Include spot in results** as shown to the right.

To move to the next stage in the workflow click **Section Complete.**

Stage 10: Multivariate Statistics on Selected Spots

The tutorial displays the **full** functionality of the Multivariate Statistics, however, only the PCA analysis functionality is available in the Standard release of Progenesis SameSpots.

For this tutorial we start by examining the behaviour of all 890 selected spots from the previous **Review Stage** then making use of the Tags and Group Setups to explore specific groups of spots that meet chosen statistical thresholds.

The statistical analysis of the selected data is presented to you in the form of interactive graphical representations of answers to questions asked of the analysed data.

For example: on opening **Progenesis Stats** the first statistically based question asked of the application is to:

Are there any outliers in my data? And does my data cluster according to my groups

It answers this question by:

 'Using Principal Components Analysis (PCA) to produce a simplified graphical representation of your multidimensional data'.

 Note: the images (samples) are displayed as solid coloured circles on the plot. To identify each image a tooltip is displayed when the cursor is held over the required circle.
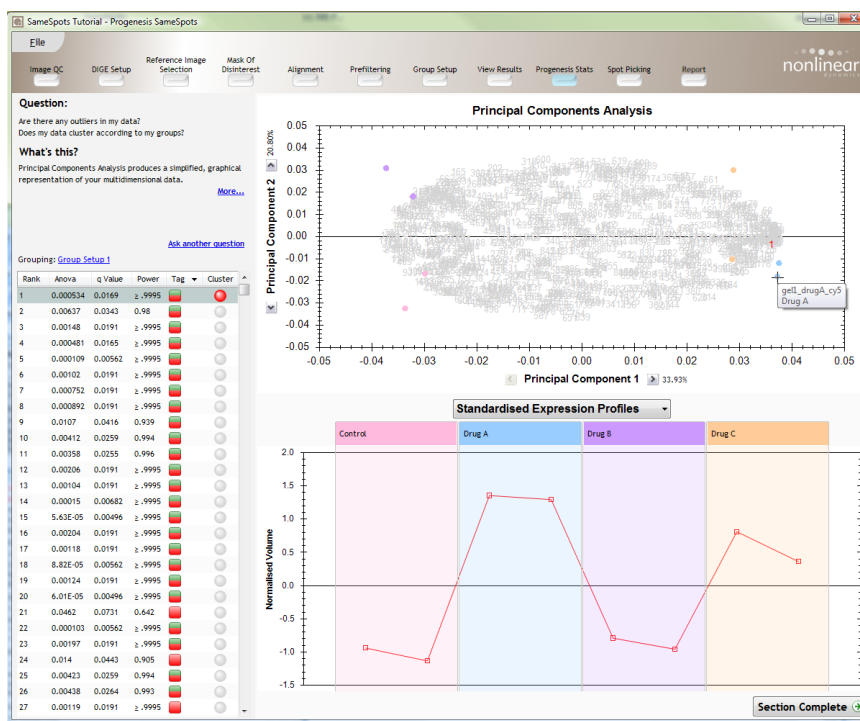

Principal Component Analysis (PCA)

PCA can be used to determine whether there are any outliers in the data and also look at how well the samples group. The groupings that can be observed on the 2D PCA plot can be compared to your experimental design groupings and conclusions can be drawn regarding possible outliers in your original groupings.

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Now examine the effect on the PCA plot when you focus on the **Top 50 Ranked Spots for All groups** by selecting the Red Tag (see below) this provides a clear picture of the relationships between the groups.
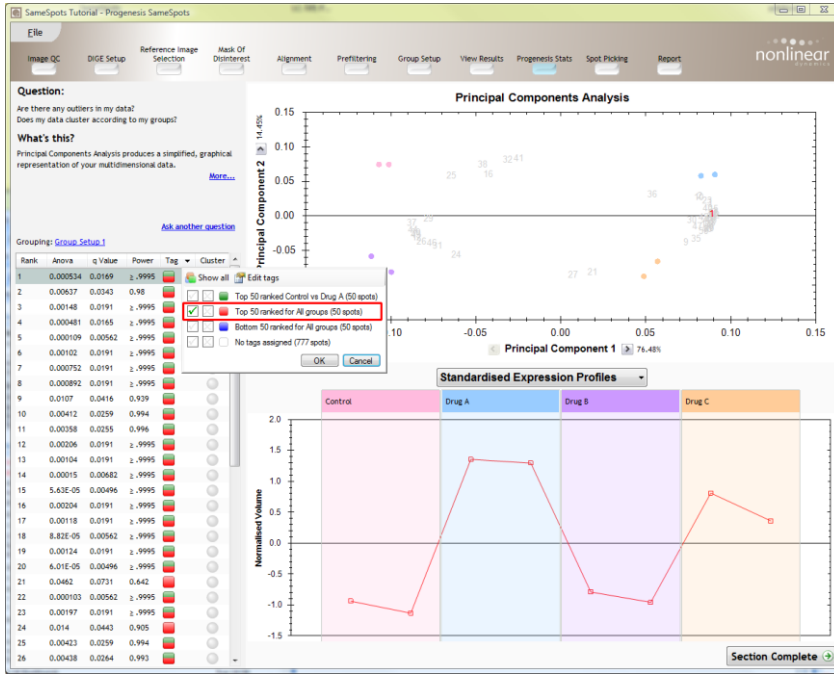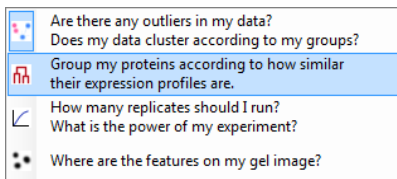

This can be compared with the Bottom 50 ranked spots (see below) which indicates no clear groupings of the sample data
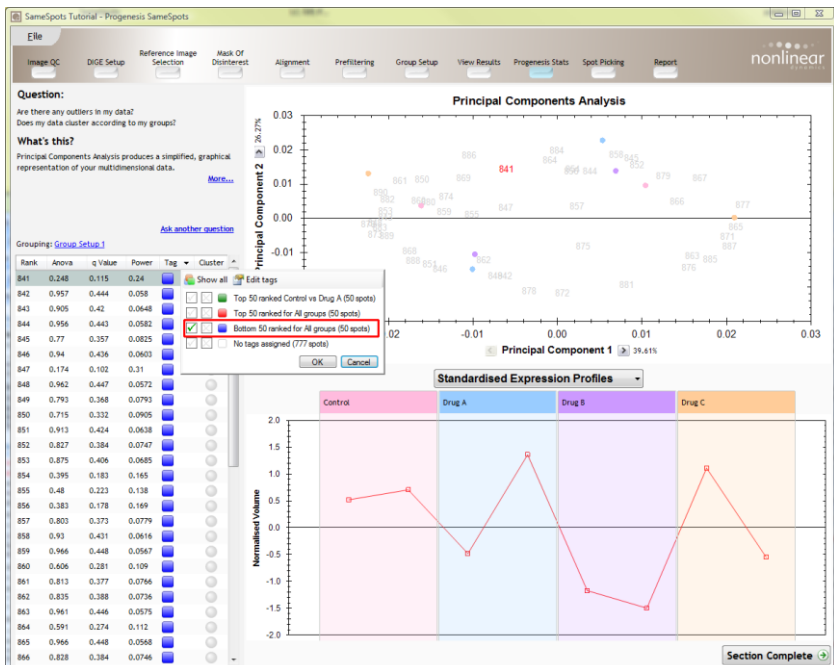
Before examining the data in Correlation Analysis **Untick** the Tag to restore all 889 spots in the PCA Biplot. Now click on the link Ask another question (above the table).

A selection of 4 tools will appear in the form of questions.

Select the second option to explore 'grouping of proteins according to their expression profiles'

This time the statistically based question(s) being asked of the application is:

'Group my (selected) proteins according to how similar their expression profiles are'

It answers this question by:

'Using Correlation analysis to evaluate the relationships between the (selected) proteins' expression profiles'.

The answer is then displayed graphically in the form of an interactive dendrogram where the distance, horizontally and vertically, between each spot can be taken as a measure of how similar the expression profiles of each spot are.

Correlation Analysis enables the grouping of proteins together according to how similar their expression profiles are. Thus showing which ones might be co-regulated and therefore probably involved in the same pathway.

Spots with a high correlation value (i.e. close to 1) show similar expression profiles while spots which a high negative correlation value (i.e. close to -1) show opposing expression profiles.

Individual spots can be selected from the table where as multiple spots can be selected from the table or by clicking on a 'node' in the dendrogram as shown below. Multiple nodes can be selected by holding down **Ctrl** and clicking on the required nodes in the dendrogram as shown below.

Additional expression profiles can be added or removed from the graph by holding down **Ctrl** and clicking on the spot entry in the table.

Now focus the dendrogram on the **Top 50 Ranked Spots for All groups** by selecting the first Tag created in the previous section.

Multiple groups can be displayed by setting a 'pruning threshold' on the Dendrogram. To adjust this threshold (horizontal grey line located at the top of the y axis) click and drag the line down over the dendrogram.
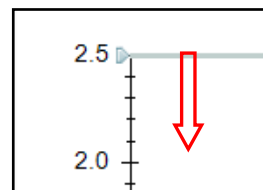
Note: as it moves over each branch all the branches beneath become colour coded and selected

When you release the mouse button the expression profiles for each branch of the dendrogram will be displayed. The number and composition of these 'branches' is controlled by sliding the threshold up and down.

To identify the shape of individual profiles in the expression graphs drag the mouse over the profiles. The current profile is highlighted with a tool tip showing the ranking and relevant sample information.

Finally, the table can be ordered based on cluster by clicking on the cluster column.
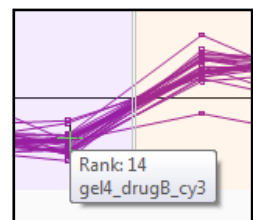
Additional tagging of spots in Progenesis Stats

The Dendrogram view can be used to further categorise and therefore Tag groups of Spots based on their expression profiles.

In the dendrogram view switch on the Tag: **Top 50 ranked Control vs Drug A** spots with 'Group Setup 1 still displayed (as shown below).
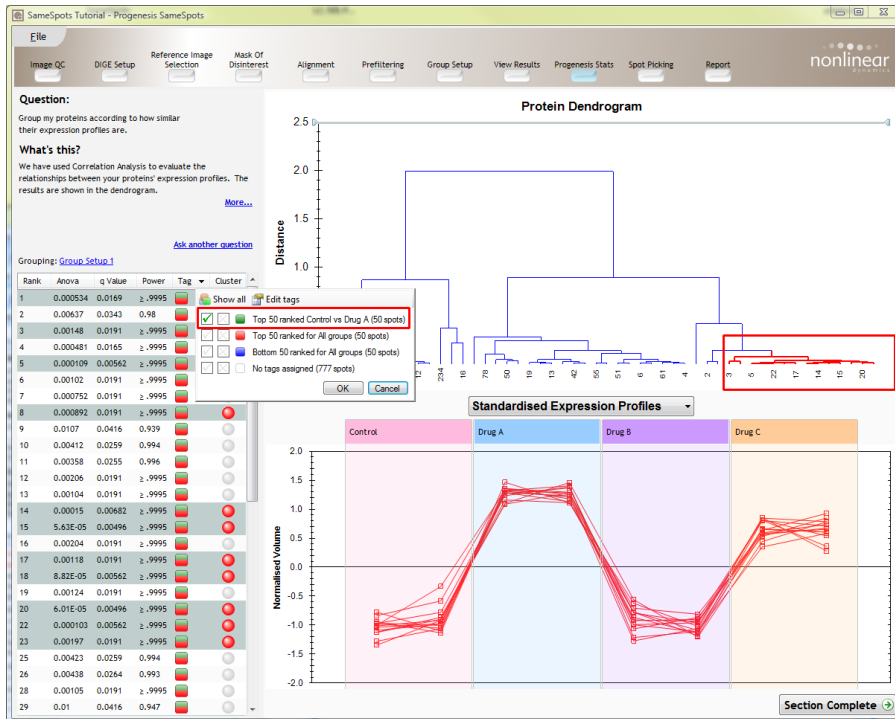
As you click on a 'branch' of the dendrogram the spot 'expression' profiles are displayed and all the spots in the selected limb become selected in the table.

To set a new Tag, for these spots with similar expression behavior, right click on a selected area of the table and select **New Tag**
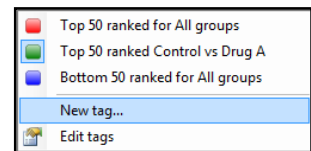
Give the Tag a name: **Pick 'up' expressed in Drug A**
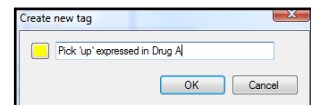
Now click on the link Ask another question (above the table).

Select the fourth option to view the location of the tagged spots on the gel image.
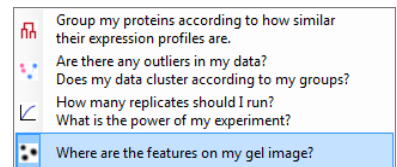
Make sure that the new Tag you have created is the only one selected
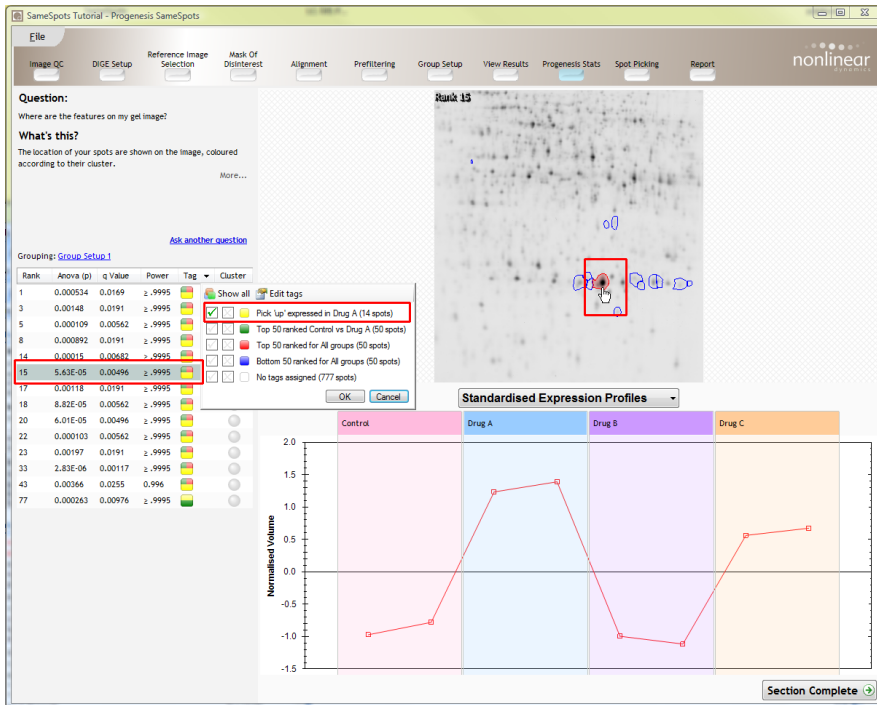
The image will display all 14 tagged spots. Click on any spot in the image to review the individual expression profiles.

To **Untag** a spot, right click on the selected spot in the table and click on the Colour of tag you require to remove.

Note: To restore/add a Tag right click on the selected spot in the table and click on the Colour of tag you require to add.
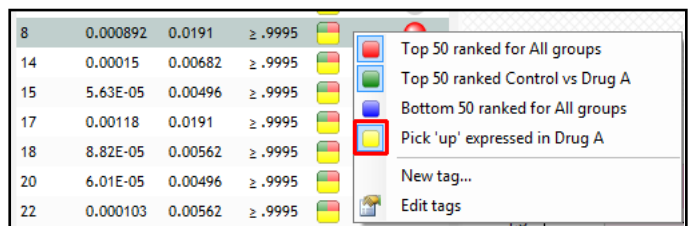
Power Analysis

To explore the third Statistical analysis of the data click on the blue link at the top of the table. The selection of 4 tools will appear in the form of questions.
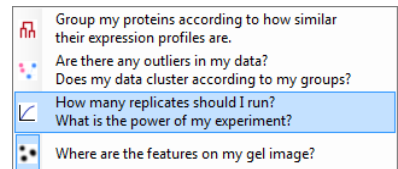
Select the third option to explore the number of replicates required and obtain a measure of the 'power' of the current experiment.

This time the statistically based question(s) being asked of the application is:

'How many replicates should I run and what is the power of my experiment?'

It answers this question by informing you:

'How many replicates your need to achieve at least 80% of your spots with a power >0.8'

Using the **Top 50 ranked for All groups** (spots) view the power analysis.

This is displayed graphically showing what percentage of the 50 spots used in the test, give a power of 80% or more and how this relates to the **number of replicates**.
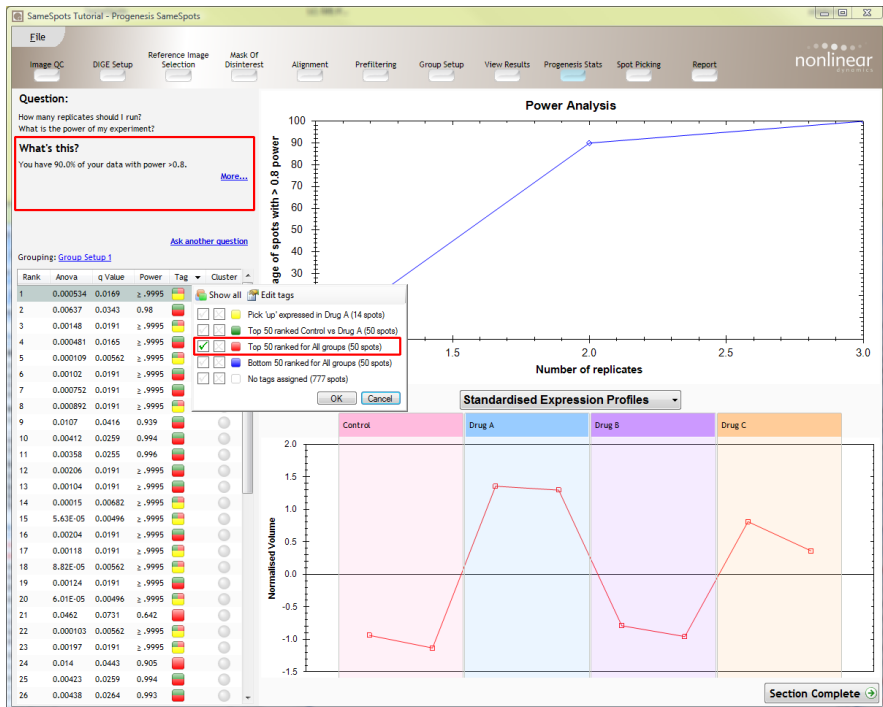
Power Analysis

- **The power of a statistical test** reflects our confidence in the experimental data's ability to find the differences that do actually exist

- The power is expressed as a percentage, where 80% power is an accepted level, therefore allowing you to assess the number of sample replicates that would be required to achieve a power of 80%.

Picking Tagged spots

Before leaving Progenesis Stats ensure that all the Tags are **Un-ticked**, then click on **View Results** in the top Workflow. Select all 889 spots and click on **Don't include spot in analysis** at the bottom of the table.

Then tick on the **Pick 'up' expression in Drug A** tag, select all spots in the table and click on **Include spots in results.**

Now click on **Spot Picking** to move on to picking your 14 Tagged spots.

> **Note**: if you are not picking then click on **Report** to generate a report on the currently selected spots (go to page 41).

Stage 11: Spot picking

At this stage in the Workflow you can set up the spot picking to pick off either one of the gels in the experiment or from an additional image run for the purpose of picking off. In the case of DIGE the latter is the norm where a gel external to the experiment is used as the picking gel.

Picking involves the alignment of one or more analysed images with the original gel images in order to export the correct picking coordinates to the robot of choice.

For the tutorial example we have selected 19 spots, corresponding to increased expression under Drug A conditions to be picked. Therefore an external image to the experiment which corresponds to a sample treated with Drug A (stained with Sypro) is used as the picking gel image.

Browse to locate the external picking image
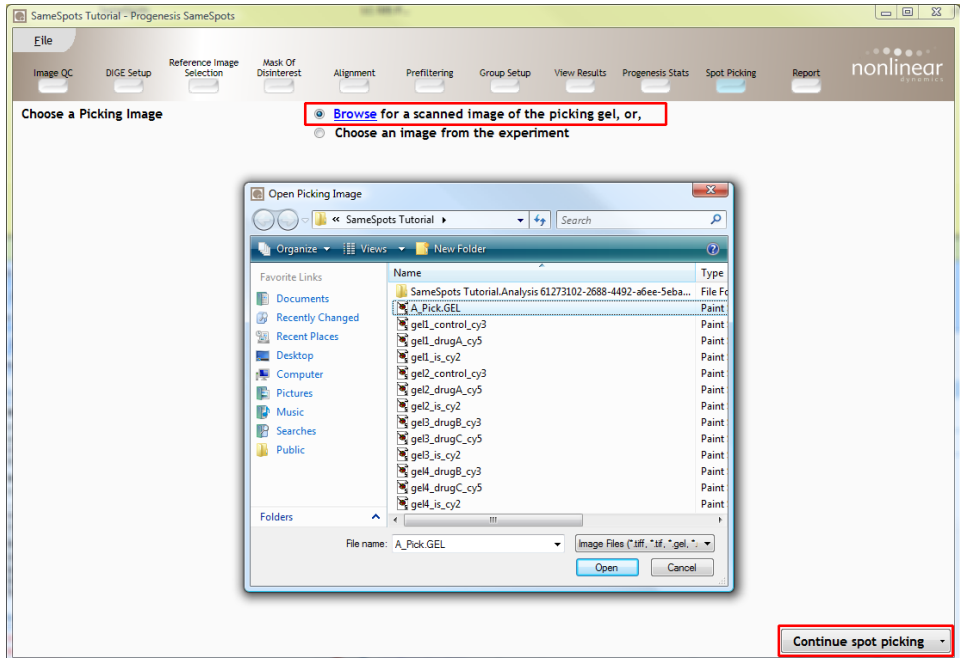
Having located the picking image, click **Continue spot picking** and select the image in the experiment that corresponds best to the picking image. In this example one of the Drug A analysis images should be chosen (see below).
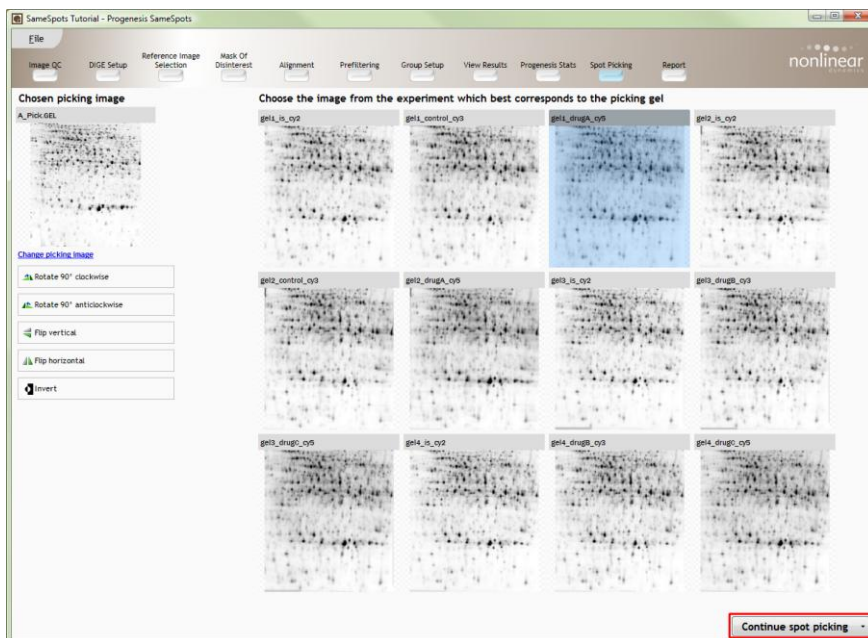
To move on to aligning the two images click **Continue spot picking.**

The application now looks like the Alignment Stage; the process of alignment is the same.

Note: because you may be aligning images captured using different Stains and also different protein loadings the performance of the Automatic vectors may require manual intervention to improve the quality of alignment in certain areas (see below).
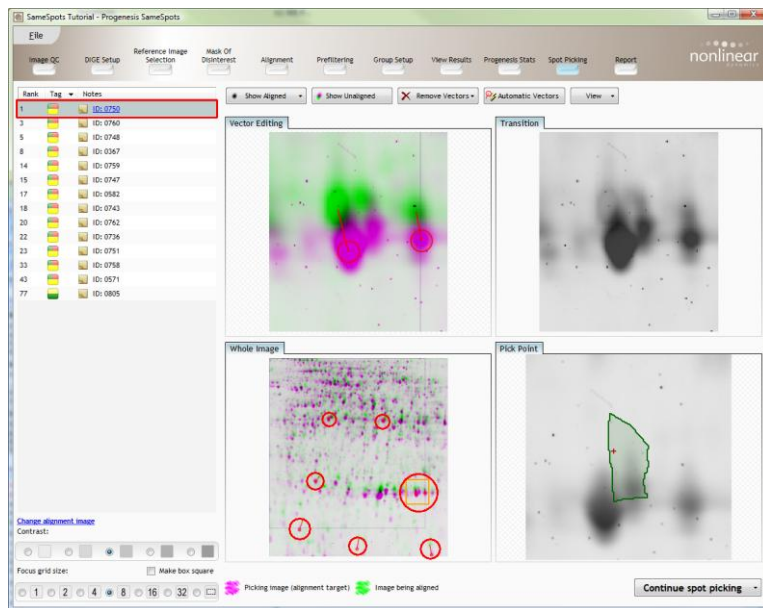
It helps to place 5-10 manual vectors (circled above) to assist the generation of the Automatic vectors. Once the manual vectors have been added click **Automatic Vectors** then confirm that the spot overlays for the picklist are correctly aligned with the spots on the picking image.

Note: the pick point (enlarge view bottom right of display) will update as the 'Show Aligned' button is pressed.

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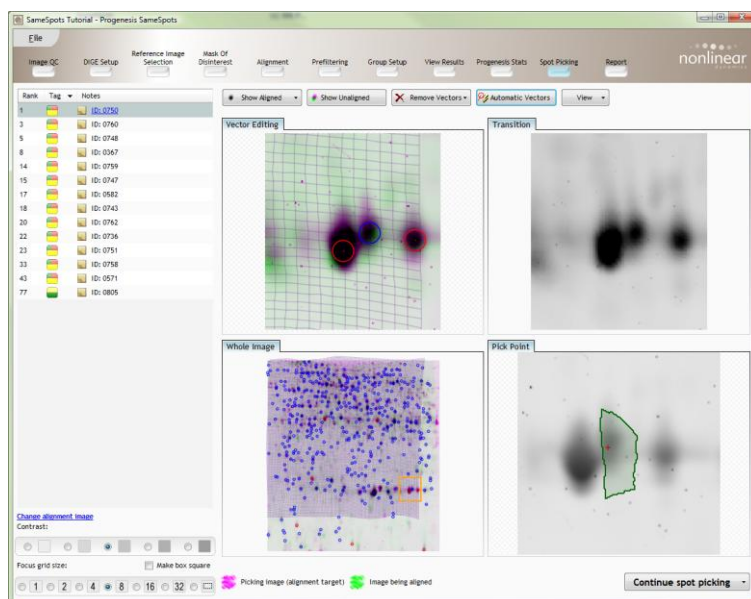
Refining alignment and Pick Point location

To refine the alignment in an area, add and remove vectors using left and right clicks and update the alignment using **show aligned**.

Note: To remove a vector right click on it; to add a vector 'left click and drag' it if necessary to position it accurately.

As an example let us examine the refinement of spot 20

Following the generation of automatic vectors the spot alignment appears unsatisfactory although the spot shape includes the area we want to pick.
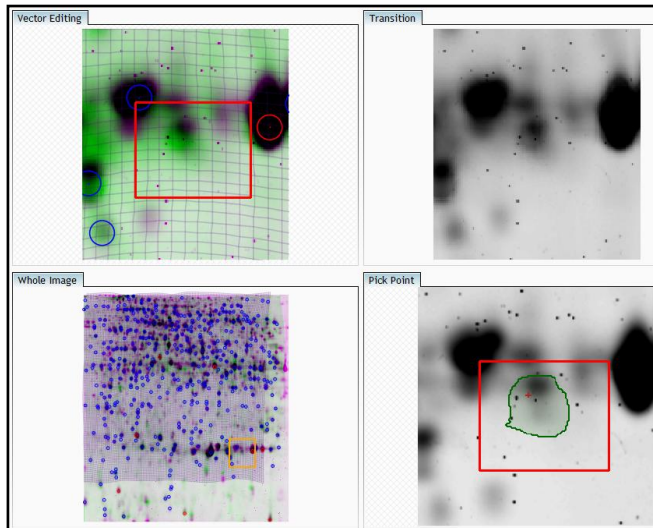
First option is to improve the alignment by manually adding a vector to the spot. Click on the spots in the 'Vector Editing' view and reposition it by dragging the spot then release it. A new (red) manual vector will appear. Press **Show aligned** to see effect of adding the vector.
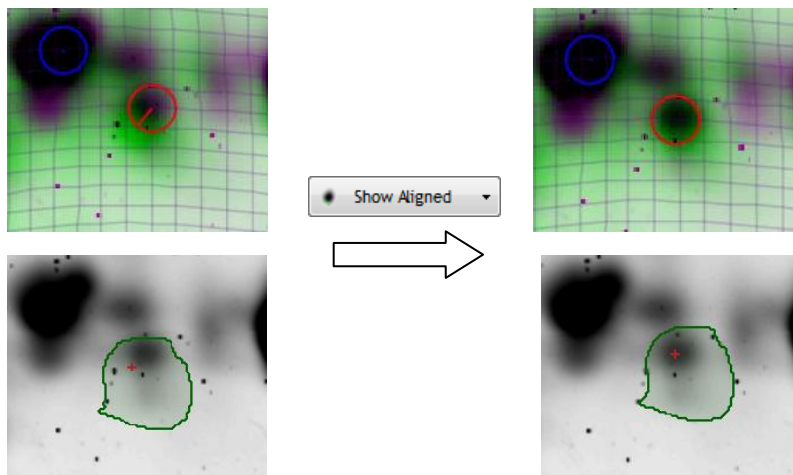
Second option is to accept the alignment but reposition the **Pick Point** (red cross on the bottom left view) by clicking on the area of maximum intensity.

Reposition **Pick Point** by clicking on a new location
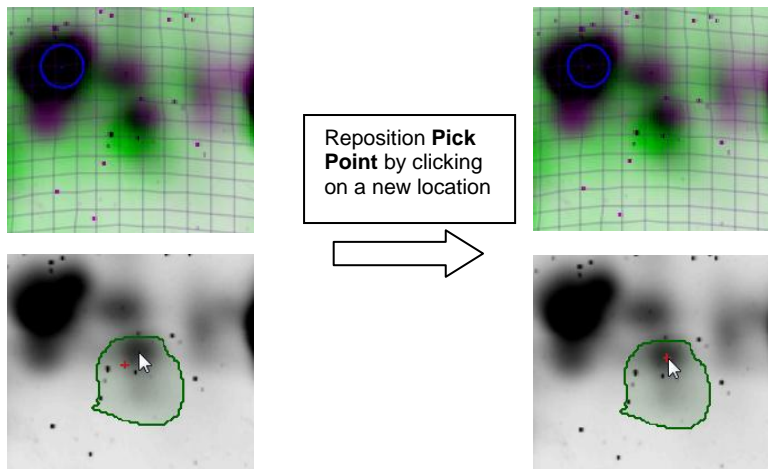
By using a combination of the first and second options you can quickly review and refine the pick list

To change alignment image in your experiment use the link at the bottom of the picklist

Combine the use of the AutoVectors and manual alignment to ensure that the 19 spots are correctly aligned.

Having refined the alignment to your picklist click **Continue spot picking**
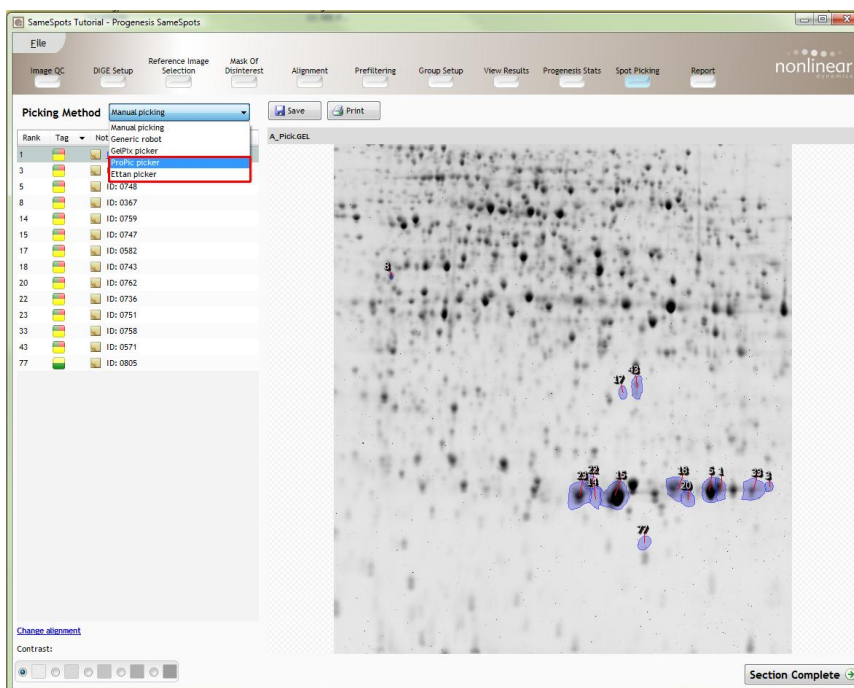
The view will display all the spots to be picked with **Picking Method** set to **Manual picking**. Set the picking method to your current robot for example, **Propic picker.** The screen will display the appropriate information as shown below which can be exported to a file for the Robot.
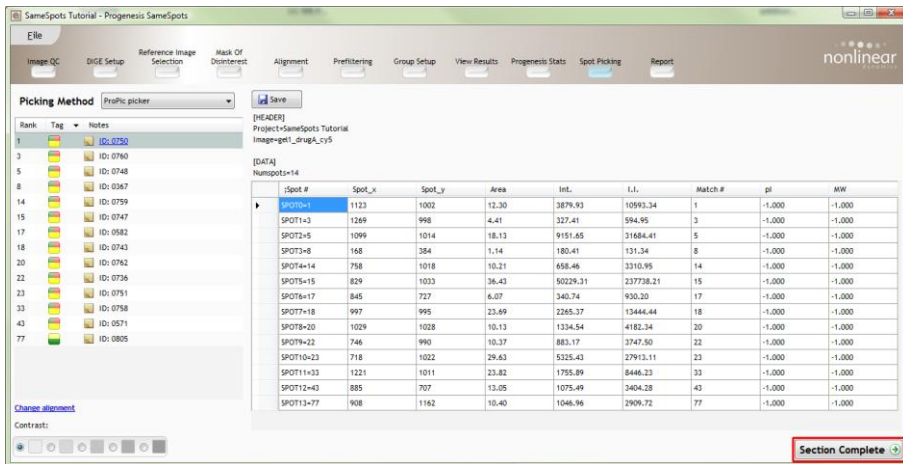
Stage 12: Reporting

The **Report Design** stage allows you to select what views you want to include in a report based on the list of **currently selected spots.**
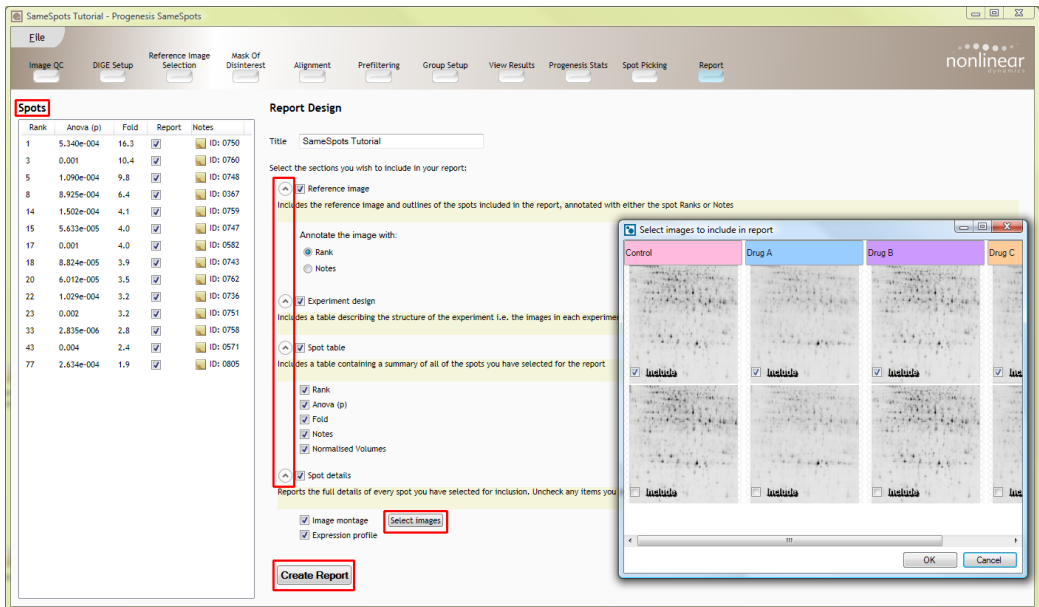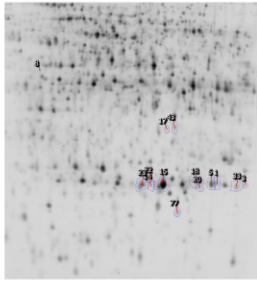
You can also control which images are displayed in the report montage views.

Click **Create Report** to generate the report.
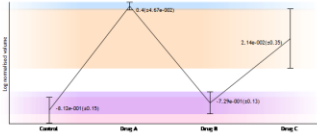
Scroll down to view details for each spot and montage of selected images. The report is printable and/or can be saved.

Congratulations!

This document has taken you through a complete analysis using SameSpots, from advanced image alignment through image analysis with minimum editing required to generate lists of interesting spots using powerful Multivariate Statistical analysis of the data.

Hopefully our example has shown you how this unique technology, currently available within Progenesis SameSpots package, can deliver significant benefits with

- Speed
- Objectivity
- Statistical Power

> If you would like to see the benefits of running Progenesis SameSpots using your own images and explore the Quality Control module as well as the rest of the SameSpots workflow please go to the next section.

Appendix 1: Licensing images (Stage 4)

When setting up a **New experiment** if you are evaluating SameSpots with unlicensed images then the licensing page will open after **Reference Image Selection**
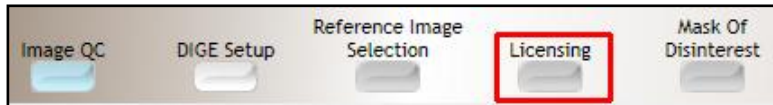

> **If you already have a programmed dongle attached to your machine then the following License Images page will not appear.**

To use this page to License your images **you must first either obtain an 'Evaluation' License Code from a Nonlinear Sales Person or purchase a license code directly from Nonlinear**.
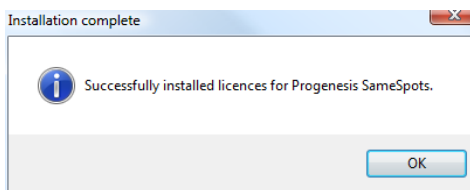
Each code will allow you to license a set number of images.

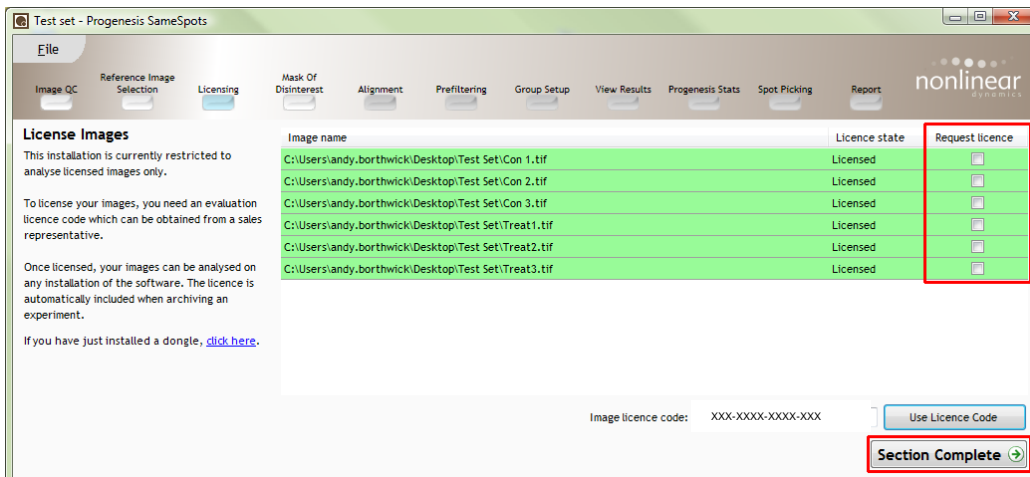For the example the images you wish to License will be listed as shown below.

To activate license(s) for the selected images enter the code in the space provided and click **Use License code.**
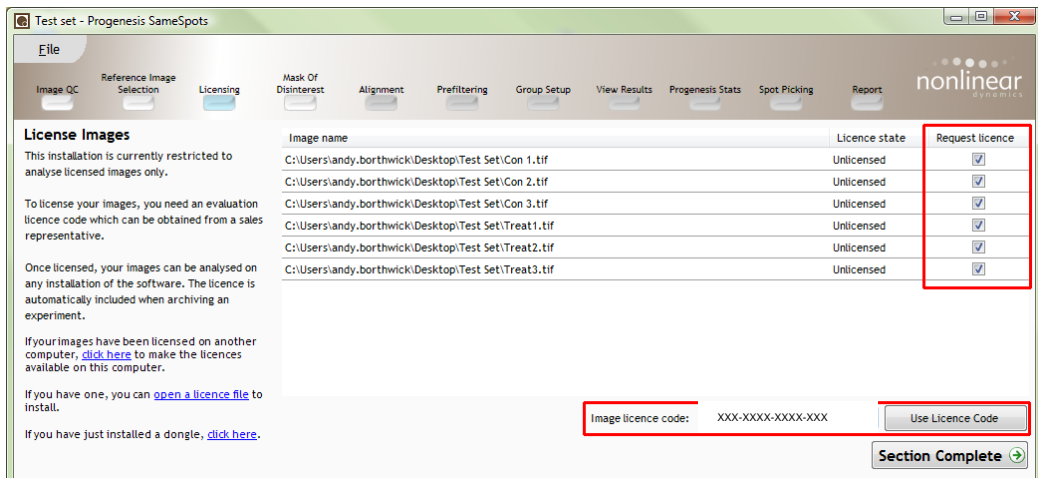
A message confirming successful installation of your image licenses will appear.

Click OK, the view will update the display to show that the images are now licensed

Appendix 2: Edit Alignment Vectors

If you need to edit then the following features will be of use.

Adding and Deleting individual vectors

Vectors, including the automatically generated vectors, can be added and deleted, in the same way you added 'seed' vectors before alignment.

Clearing vectors

Groups of vectors can be deleted from the focus area by using the appropriate drop down option from the **Remove vectors** tool bar button.
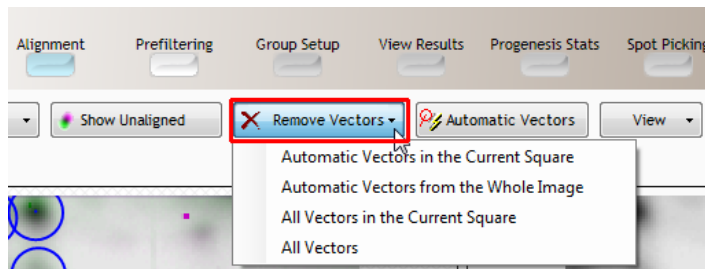
Displaying Alignment

Just as the **Show Aligned** button switches on the aligned view of the images then the **Show Unaligned** button on the top tool bar switches back to the unaligned view that may help when editing vectors.

Re-calculating auto vectors

Pressing the **Automatic vectors** button again after editing will use **All** the vectors in the image, including previously generated automatic vectors, to repopulate the image. This will tend to fill in the areas cleared by the user.

Note: if auto vectors already exist on an image then you will need to tick the box to make it create more.
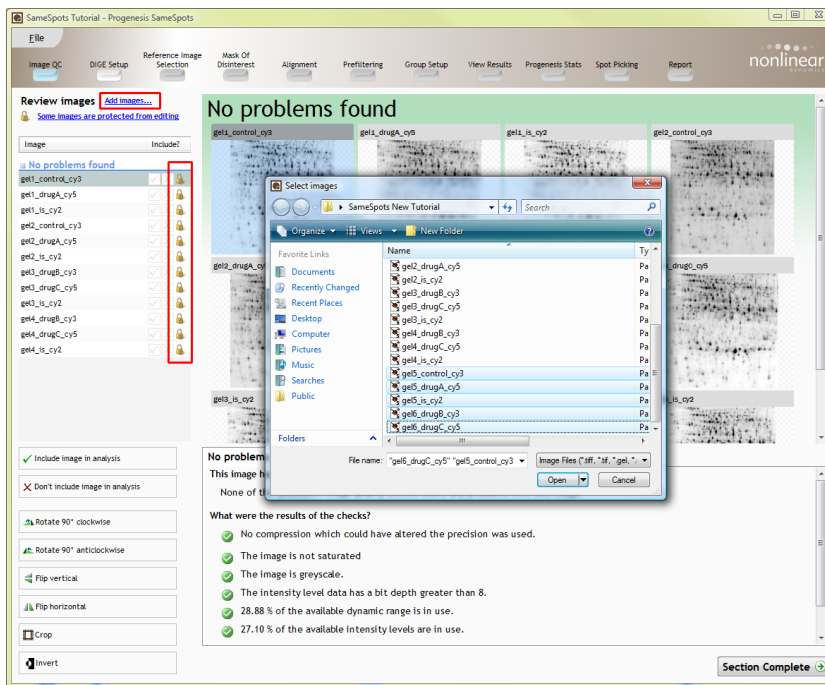
Appendix 3: Adding images to an existing experiment

To add additional images to an experiment that has already been analysed, and perform SameSpots analysis on the new images you must return to Image QC.

Click on ImageQC on the workflow

Note: for best results the new images should relate to the existing Reference gel and experimental design.

**Note**: as Image QC opens the existing images will be locked.

The new images will NOT be locked and you can adjust them (crop etc) if required.

Having added the new images assign them to the correct gels at the DIGE setup.

The Reference image and Mask will also be locked (see blue link), leave them locked and move forward to the Alignment stage.

Align the new images as described in this tutorial (page 12) and click on Prefiltering to Analyse.
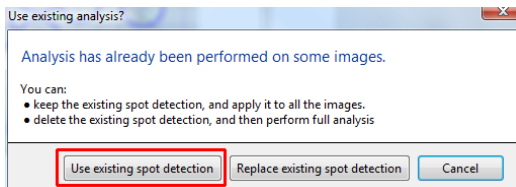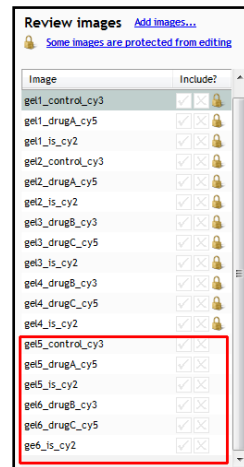
Note: before Analysis takes place you get the option to use the existing spot detection or replace it with a new one which takes into account additional information from the new images.