SameSpots DIGE Tutorial

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Introduction to the Tutorial

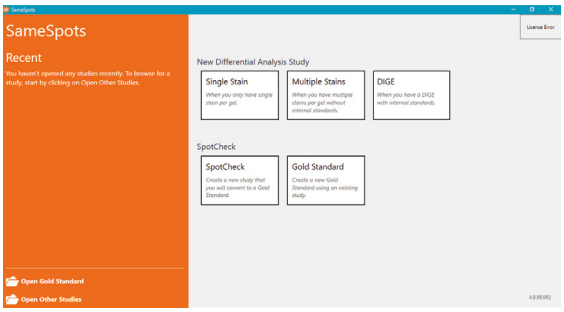
Using this tutorial, you will learn the basic workflow of analysis within SameSpots. Although it will not go into depth on the various features, it will help you become familiar with the steps required to identify the interesting spots in your experiments.

The data used for the tutorial is taken from a DIGE experiment. If you don't normally use DIGE, there is also a single-stain tutorial available.

About the experiment

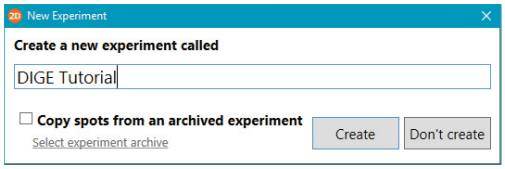
The data you'll use in this tutorial is a subset of gel images from a study into the effects of three different drug candidates. It consists of 12 images, taken from a total of 4 DIGE gels, each gel running 2 experimental samples (cy3- and cy5-stained) and an internal standard (cy2). Consequently, each condition under test — control, treated with Drug A, treated with Drug B, or treated with Drug C — is represented here by only 2 replicates. It's important to note that this is not recommended for real-world experiments. We've used a small dataset here to keep the download size to a minimum, while still allowing us to demonstrate the key features of the software.

Creating the experiment



Once the images have been downloaded, we'll begin our analysis by creating a new experiment. If it's not already open, launch SameSpots and then:

1. Start a **New Differential Analysis Study** by selecting the **DIGE** option.
2. In the **New Experiment** window, enter the name "**DIGE Tutorial**" in the text box.
3. Leave the **Copy spots from an archived experiment** unselected.
4. Click the **Create** button to create the new experiment.

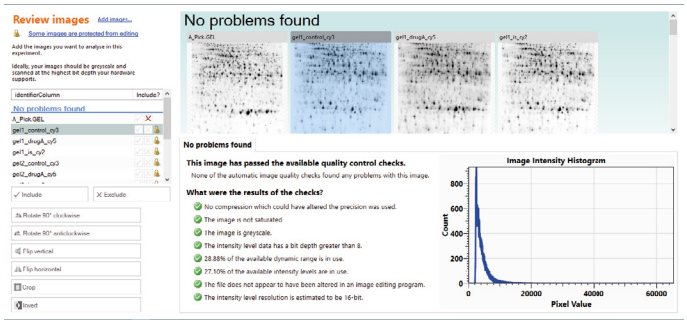


SameSpots then opens the new experiment, putting us at the start of the main analysis workflow, ready to import our gel images. Let's get started…

Importing and aligning gel images

As with any analysis in the main workflow, the first step is to import your gel images. The experiment used in this tutorial explores changes in protein expression between three drug treatments (A, B and C) and a control. The samples were run across 4 separate gels and, as this is a DIGE experiment, each of those gels generated 3 images. This gives us a total of 12 images to add to the experiment:

1. Click the **Add images** link at the top-left of the screen, or the **add some images** link in the centre of the screen.
2. Select the image files in the folder to which you copied the tutorial images earlier.
3. Click **Open**.

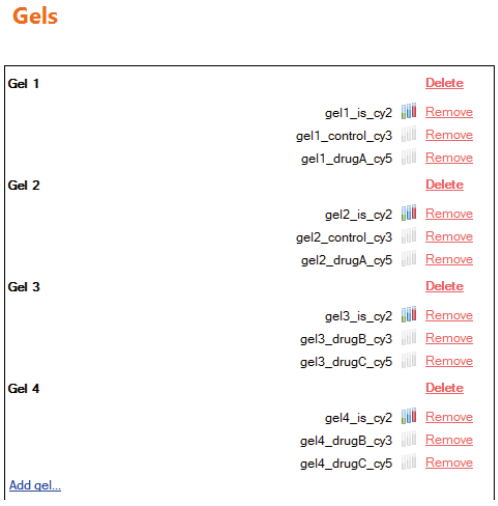


Images are automatically quality checked when they are uploaded to the software. There are no quality issues with these images, move on by selecting **Next step** in the top right corner.

DIGE setup

The **DIGE Setup** screen, ready for us to regroup the images by physical gel. In our case, the image files have been labelled well enough that grouping them is made simple; all grouping can be performed using the filter box above the gel images:

1. Enter the text **Gel1** into the filter box above the gel images, or click on each image of **Gel1** to select them.
2. Click the **Add Selected Images To Gel** button to create the first DIGE gel in the list at the left.
3. Repeats steps 1 and 2 above, for **Gel2**, **Gel3** and **Gel4**.



SameSpots will automatically detect which images are the internal standard images. Once complete, your list of DIGE gels should look like this. When you're ready, click **Next Step** to move to the next screen.

Aligning the gel images

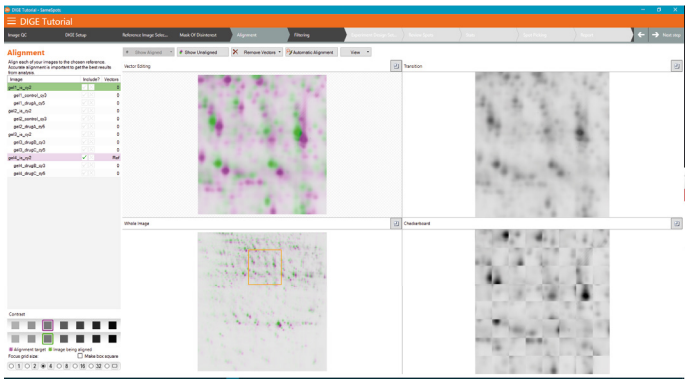
Alignment of gel images is a critical step in any analysis, allowing us to perform accurate and statistically robust analysis of your data. Before we can align, however, the **Reference Image Selection** screen allows us to define a representative gel image to which the others will be aligned.

For this experiment, we'll use the internal standard of **Gel 4** as the image to which all others are aligned. This is automatically selected as the reference image, so we can again click **Next step** to move to the next screen. Sometimes when you capture images of your gels, you may include areas that don't contain any of your test samples e.g. MW calibration ladders. These areas can be excluded from the analysis — masked out — using the **Mask of Disinterest** step of SameSpots.

For this tutorial, however, all gel images have been cropped well, so we don't need to apply a mask. Instead, click the **Next step** button again, to move on to the **Alignment** screen.

What does the alignment screen show?

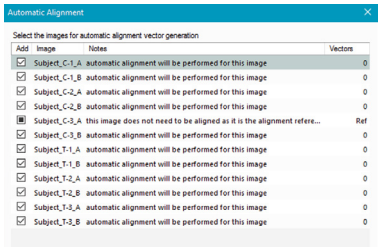
The alignment screen is designed to make finding and fixing any alignment problems as easy as possible. Each of the 4 main views shows a combination of the same two images: the image being aligned (in green, selected in the list at the left); and the reference image (in magenta, image **gel4_is_cy2** in this example).



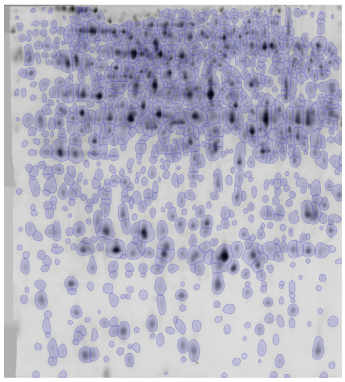
By moving the rectangle in the **Whole Image** view (using cursor keys or mouse), different areas of the gel are shown in close-up in the other views. The green spots of the alignment image can be manually dragged onto the corresponding pink spots of the reference in the **Vector Editing view.**

For your own experiments, it's worth spending some time learning about manual alignment, but for speed in this tutorial, we'll use only the automatic alignment option:

1. In the **Alignment** screen, click **Automatic Alignment**. In the window that appears, make sure all images are selected (except the reference image, **gel4_is_cy2**).
2. Click OK to begin aligning the images.
3. Once the auto-alignment is complete, click **Next step** to continue.



The **Detection Parameters** window pops up. Check all images are selected then click **Detect**. Once the spots have been detected, we see the results in the **Filtering** screen.

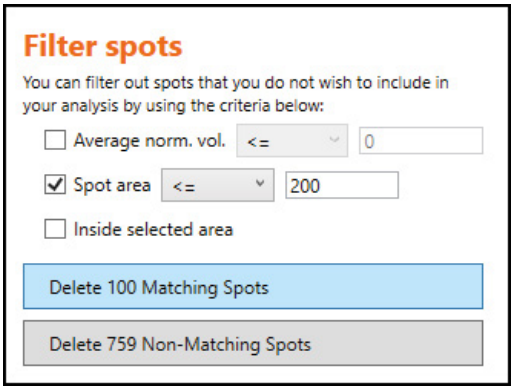


Each of the outlines shown is a spot and that region is quantified on each of the aligned images. This means that every image has a measurement for every spot — it's this that allows us to produce robust statistics, avoiding the problems associated with missing values.

Removing unreliable spots

Filtering allows us to remove spots whose quantification we can see will be unreliable. For example, any spots detected in damaged areas of the gel could be discarded. The gel images in this experiment, however, have been run and captured well. We'll restrict our actions on the **Filtering** screen to removing spots that may be too small to pick reliably:

1. Select the **Spot area** check box.
2. Leave the ≤ option selected in the check box's associated drop-down list.
3. Enter a value of **200** in the associated numeric field.
4. With the filter now ready to apply, click the **Delete 100 Matching Spots** button.



When analysing your own images, you may need to use different values for the spot area if applying the same kind of filter. This is because it depends upon the resolution of your images and the size of spot you can reliably pick (which is often determined by the head size of your picking robot).

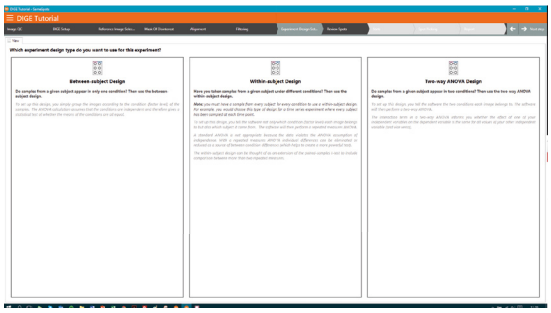
For this tutorial, however, we are ready to continue our analysis. Click the **Next step** button; spot measurements are then normalised across our samples automatically and we can begin investigating the protein expression differences.

Identifying interesting behaviour

To investigate the changes happening between our samples, we need to define which gel images represent which experimental condition. This allows us to directly compare protein expression in different conditions, analysing the samples in those conditions as a whole.

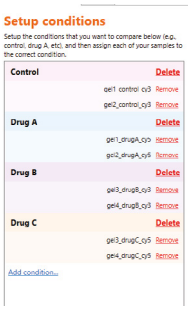
The **Experiment Design Setup** screen helps us to do this.

First, we must specify which type of experiment design we're using. In this example, each sample analysed comes from a different subject and there is no time-series and each sample comes from only one condition.



This means we should build a between-subject design:

1. Click on the **Between-subject Design** panel.
2. In the window that appears, enter a name of **"All conditions"** and click the **Create design** button.
3. Select the images which represent the controlled condition by typing **"Control"** into the filter box or clicking on each image with **Control** in the name.
4. Click on **Add Selected Images to Condition** to create the first condition in the list to the left.
5. Repeat steps 3 and 4 for **Drug A, Drug B** and **Drug C**.

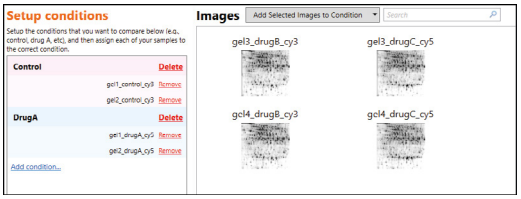


With SameSpots, it's possible to define multiple designs in a single experiment. This gives us the flexibility to compare our samples in many ways. We'll show this now by creating a second experiment design that will allow us to focus on the effects of Drug A:

1. Select the **New** tab in the **Experiment Design Setup** screen.
2. Once again, click on the **Between-subject Design** panel.
3. Enter a name of **Control vs. Drug A** for the new design.
4. Select the **Copy layout from** option and leave the value in the drop-down list set to All conditions.
5. Click **Create design** to create the new design. You'll notice that, this time, our new design is already set up, exactly the same as the All conditions design.

You'll notice that, this time, our new design is already set up, exactly the same as the All conditions design.

To compare the **control** condition to **Drug A**, we need only the first two conditions; click on the red Delete links at the right of the **Drug B** and **Drug C** conditions. The design will now look like this:



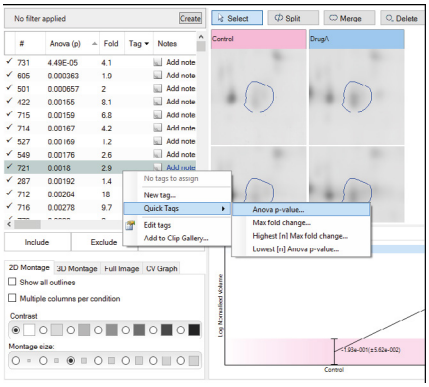
We're now ready to compare the expression of proteins between the control samples and those treated with Drug A. Make sure you still have the **Control vs. Drug A** tab selected, then click **Next Step** to move on to the View Results screen.

Finding significantly changing spots

The **Review Spots** and **Stats** screens help us to find the interesting spots and explore our data in a variety of ways. The simplest way is to apply tags to spots based on their measurements. We can then filter our data to show only those spots with particular combinations of tags.

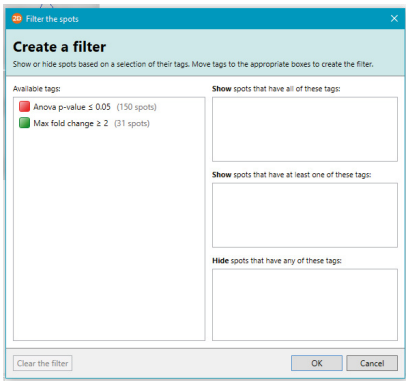
While the **Review Spots** screen offers many powerful visualisations of our data, the particular part of the screen that we need for tagging is the list of spots at the left. Let's start by tagging all spots that are showing statistically significant fold changes between our experimental conditions:

1. Right-click anywhere in the list of spots.
2. From the pop up menu that appears, from the **Quick Tags** submenu select the **Anova p-value** option.
3. When prompted, accept the default settings of **Anova p-value** ≤ **0.05**, name and colour for the new tag by clicking **Create tag**.
4. Repeat steps 1 to 3 above, but from the **Quick tags** submenu select the **Max fold change** option and accept the default settings of **Max fold change** ≥ **2**, colour of the tag and name.



We've now tagged spots based on 2 separate criteria. To find spots that share both of these characteristics, we create a filter:

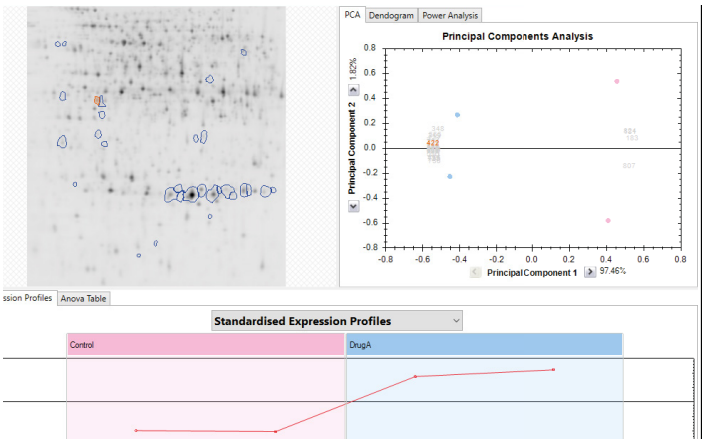
1. In the grey filter panel above the list of spots, click the **Create** button.
2. In the **Create a filter** window, click and drag each of the tags from the list at the left to the **Show spots that have all of these tags** box at the top-right.
3. Click **OK** to apply the filter.



The spot list has now been filtered to show only those whose expression has significantly changed between our control and treated samples; at least, according to our criteria.

Exploring expression patterns

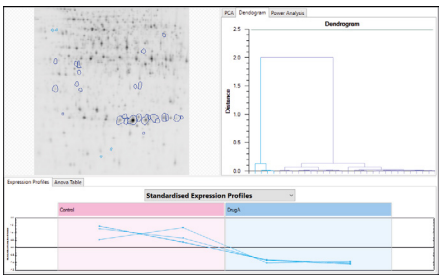
Suppose we now want to find which of our interesting spots have been up-regulated in the treated samples, with the intent of identifying them using mass-spec. There are many ways we can accomplish this in SameSpots, but we'll look at one of the more interesting approaches for exploring our data: using correlation analysis to find spots whose expression patterns are similar:



Click **Next step** to take us to the **Stats** screen. As we enter the new screen, a Principal Components Analysis ("PCA") is performed automatically. PCA can be used to determine whether there are any outliers in the data and also look at how well the samples group. Spot locations and an expression profile for selected spots is also presented.

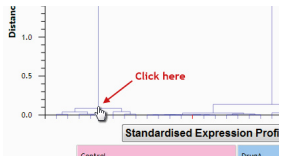
To analyse the correlation analysis, click on the tab which is titled **Dendrogram**. A dendrogram is then presented, showing how similar the spots' expression profiles are. We can interact with this dendrogram to explore the expression patterns across our experimental conditions and the samples within them:

1. Click near the base of one of the 2 main branches. Notice that all spots represented in that branch are selected and their expression profiles shown below the dendrogram. As we can see, they all share a similar expression profile.



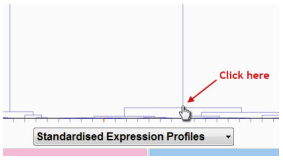
2. Now click to select the other main branch. Again, all spots represented in the branch are selected and we can see that they share a similar expression pattern, but that it is the opposite of the first branch's pattern (down-regulated vs. up-regulated, or vice versa).

3. Select the branch in which the spots have their highest expression values in the **Drug A** condition.



4. Being careful not to alter the selection, click the drop-down arrow in the spot list's **Tag** column and select the **New tag**... option. You may have to scroll to see the **Tag** column.

5. In the **Create new tag** window, enter a name of **Up-regulated** and click **OK**.

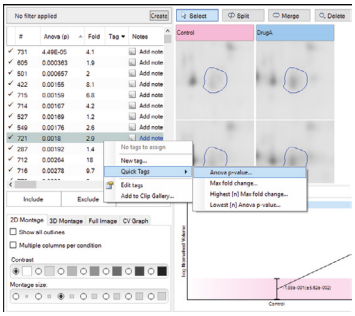


6. Now that we've tagged the up-regulated spots, we can filter our data to show only those spots:

7. In the **Tag filter** box, click the **Edit**... button to edit the filter.

8. When the **Create a filter** window appears, click the **Clear the filter** button at its bottom-left corner.

9. Now click and drag the **Up-regulated** tag to the top-right box and click **OK** to apply the filter.



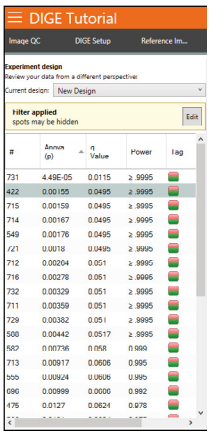
We have now identified the spots whose proteins are being up-regulated by Treatment, and we're almost ready to create a report of the analysis. First, however, we should check that the spots will yield reliable protein identifications when picked...

Producing a Picking List

Before we go ahead with protein identification, we should probably limit the number of spots that we're going to pick. For the purposes of this tutorial, we'll restrict the number of spots we aim to identify to just 10. We'll concentrate on those showing the greatest change between our control and drug-treated conditions:

To find the spots showing the greatest expression change, we'll go back to the **Review Spots** screen.

Sort the list of spots in the **Review Spots** screen by fold value by clicking on the **Fold** column header until the list shows the largest fold changes at the top.



Tagging the spots to be picked

To simplify reporting, we can mark the target spots with another new tag:

1. Making sure the list is scrolled to the top, select spot the first spot in the list of spots.
2. Hold down the **Ctrl** key and click the next spot in the list. Both spots highlighted will now be highlighted.
3. Continue down the list, holding down **Ctrl** and clicking spots until you have 10 spots selected.
4. Right-click in the list on any of the selected spots and choose the **New tag**... option from the popup menu.
5. Enter the name **Pick list** in the **Create new tag** window and click **OK**.

The spots are now tagged, so now we can filter our data accordingly:

1. In the filter panel, click the **Edit** button.
2. In the **Create a filter** window, click the **Clear the filter** button, then drag the **Pick list** tag to the top-right box.
3. Click **OK** to apply the new filter.

With our picking list ready, we can now create an html report of our analysis. Skip to the **Spot Picking** step in the workflow header to begin.

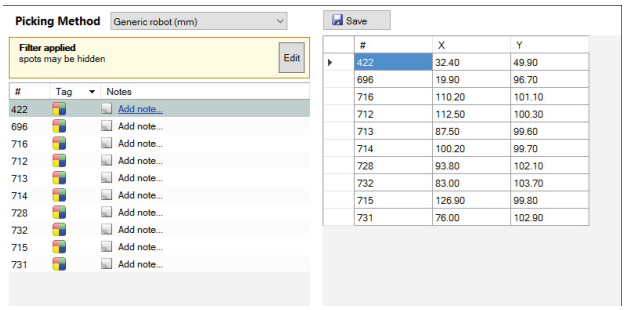
Picking spots with a generic robot

For this tutorial, we'll pretend that we own a picking robot that accepts spot coordinates in millimetres. Given this, we'll be using a gel image captured from a separate picking gel, run specifically for the purpose. On entering the Spot Picking screen, the first thing we need to do is select this image:

1. Select **Using a picking robot** option.
2. Click the **Browse** link under the **Using a picking robot** heading.
3. In the same folder as the other images for this tutorial, select the **A_Pick.GEL.tif** file and click **Open**.
4. Back in the main screen, click the **Continue spot picking** button at the bottom-right.

The next step in creating a picking file for our robot is to determine the locations of our interesting spots on the picking gel itself. This is achieved by aligning the picking gel to one of our analysis images. Given that we're interested in proteins whose expression is highest in samples treated with Drug A, our picking gel sample has also been treated with Drug A. Consequently, it makes sense to align it to an image of a Drug A sample:

1. Click on the **gel1_drugA_cy5** image in the list of analysis images.
2. Click the **Continue spot picking** again.



Now we need to align the picking gel to its reference:

1. Click on the **Automatic Alignment** button.
2. When automatic alignment is complete, click the **Continue spot picking** again.

Finally, we need to specify that we're using a robot; and select the type of robot we're using:

1. From the **Picking Method** drop-down list, select the **Generic robot (mm)** option.
2. Click the **Save** button above the table of spot coordinates.
3. In the Save picking file window, enter a name of **pick list.csv** and click **Save**.

And that's it! The picking file could then be used with the robot software to direct its picking subsequent mass spec-based protein identification.

Disclaimer

All material in this brochure has been written by collating information from various sources. Where possible these sources have been cited. It is a guide and not a protocol or standard operating procedure. It may not give optimal results for individual samples and systems. You should check parameters specific to your own sample, instruments and image capture software. Common best practice is to run pilot experiments to optimise sample handling, gel running, image capture and image analysis.

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