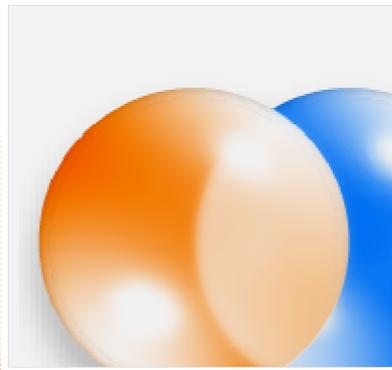
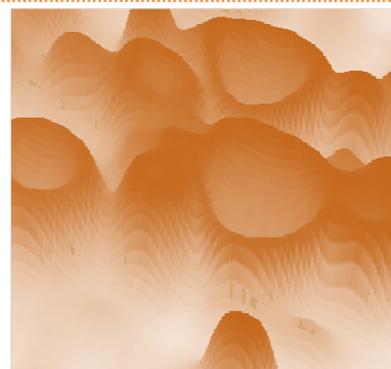
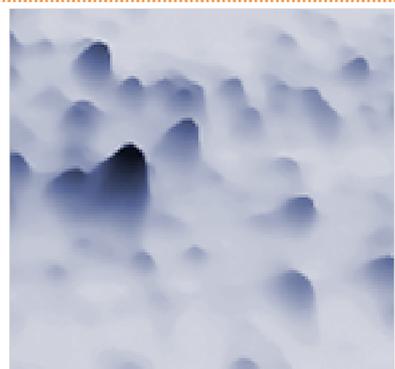
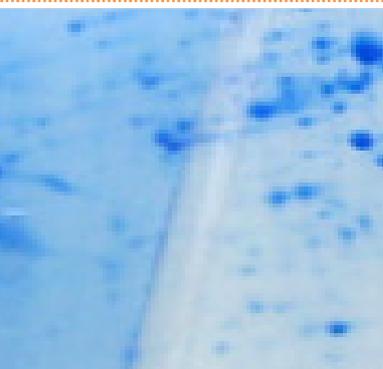
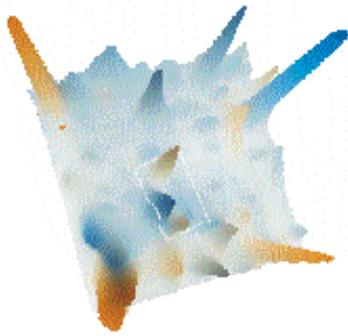


Know
ledge

Imaging 2D gels

ESSENTIAL TIPS AND TRICKS





Introduction

2D gel electrophoresis is an extremely valuable but also demanding technique for performing global proteomic analysis. Making your own gels takes time and needs hard work. After weeks of adapting the sample preparation setup, optimizing the gel running protocol, choosing the corresponding gel staining technique, and separating the samples, the final step on the way to computerized analysis is the generation of images from your 2D gels by using the adequate imaging technology.

The imaging step is at least as important for the successful analysis of your experiment as each of the previous steps.

This guide has been created to help you preserve the information that is contained in the gel and produce the best possible input for computerized 2D gel image analysis with modern software technology, as included e.g. in Delta2D¹.

1 - <http://www.delta2d.com>

2D Gel Imaging: Checklist

Here is our checklist for creating 2D gel images. We believe that it helps to achieve the best possible input for computerized image analysis. Each item is described in more detail on the following pages.

- Create **grayscale instead of color** images.
- Try to **span the complete available grayscale range** for each dye you use.
- Choose the **image resolution** such that the smallest **spots** you want to analyze **have a diameter of at least 5 pixels**.
- Process all gel images using the **same orientation**.
- Place **each gel at the same position** on the imaging device. If possible, only create an image of a region of interest.
- Use the **same parameters for all gels** in your experiment to make the imaging process easy and reproducible. **Save and reuse profiles** that include these parameters and your region of interest.
- For **multiplex imaging** adjust the parameters for each dye separately.
- **Limit post-processing** of images to crop, mirror, and rotation by 90, 180, or 270 degrees.
- Use the software for image post-processing that came with your imaging device. **Avoid third party software** like Photoshop for image processing if you do not exactly know the effects on quantitative analysis.
- **Never use JPEG files** for quantitative analysis.
- Avoid TIFF files if you can create and process **calibrated image file formats** such as GEL or IMG/INF.

Do you know any tips or tricks to be included in the next version of this guide?

Please send suggestions, questions or comments to support@decodon.com.

Which Type of Imaging Device?

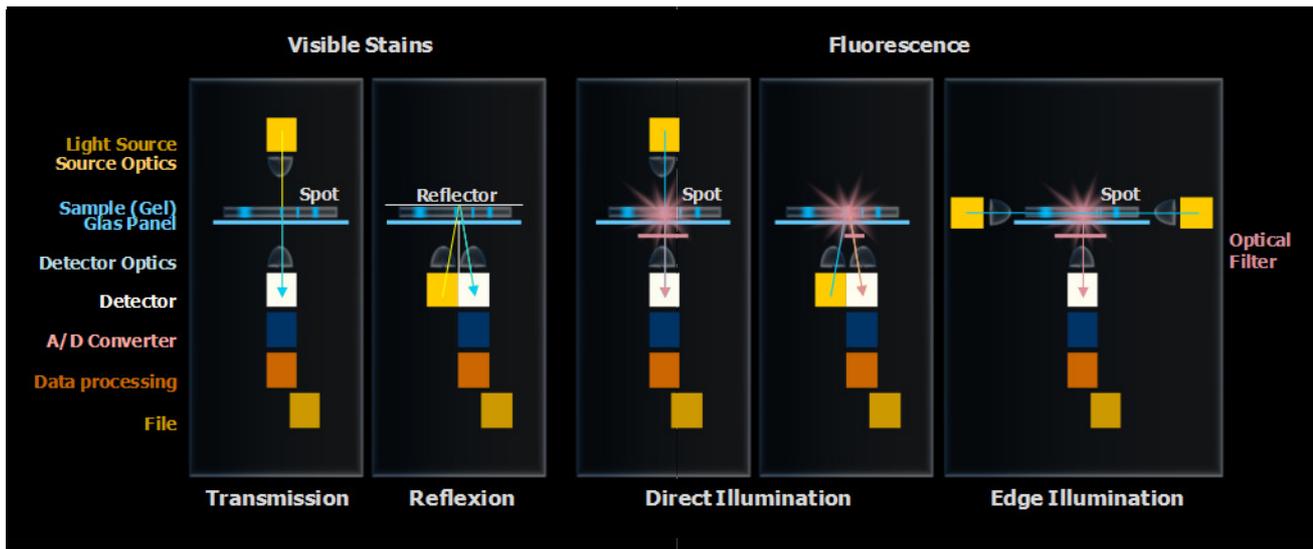


Scientific scanners and cameras are complex instruments. The following section provides some background information to make the right choices for imaging your gels.

The mode of operation of an imaging device is simple: light is emitted from a light source, transformed by the gel, and then the resulting light is measured by a detector. The signal intensity is measured for each position on the gel and converted into a number by an A/D (analog to digital) converter. The numbers are subsequently processed into a final image file.

The imaging mode indicates how the light travels from the light source to the detector (see figure on next page):

- For visible stains, in **transmission mode** the light passes through the gel and is directly measured by the detector on the opposite side of the gel. In **reflection mode** the gel is located between the light source and a reflector. Light travels through the gel, is reflected and again traverses the gel before it eventually is detected.
- Fluorescence dyes are excited by light with a particular wavelength/energy level and emit light with specific longer wavelength/lower energy level. Excitation is achieved either by **direct** or **edge illumination**.



Imaging principles for visible light (transmission, reflection) and fluorescence.

For high quality data we recommend transmission mode because saturation effects are less likely than with reflection mode. Saturation means that differences in higher spot intensities are not properly resolved - dark spot centers become completely black. In reflection mode this happens more easily because the light travels through the gel twice, which increases the likelihood of complete absorption and saturation effects (see section "Saturation Effects" for details).

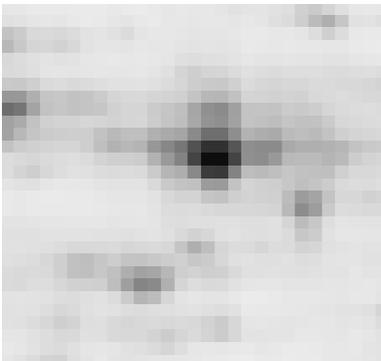
Consideration of the scan mode is important with light-absorbing stains, e.g. all types of Coomassie or silver nitrate. For fluorescence scanning the scan mode is less relevant because the detected light is emitted by the proteins within the gel.

Subsequent manipulation of the image data by using image processing software not designed for quantitative image analysis may ruin your images. Visually enhanced images often show truncations in the high and low intensity regions. Gel image files are not only pictures but also collections of quantitative data. Only flipping, mirroring, rotating in 90 degree steps and cropping leave the original data intact. Free rotating, enlarging or reducing, down scaling, any kind of gray level, contrast or gamma adjustment distort the quantitative data and may damage your image files (also see section "Data Reduction and Image Calibration").

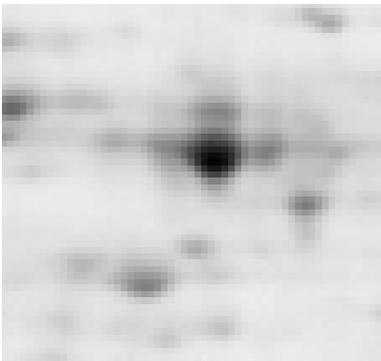
Image Resolution



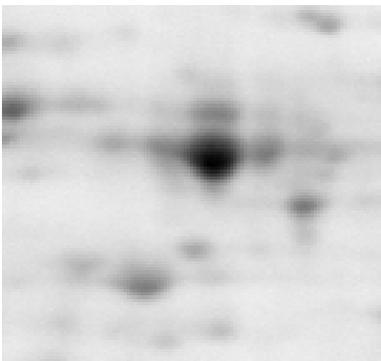
During the imaging process the 2D gel is decomposed into a mosaic of equally sized squares (pixels). Smaller pixels mean higher image resolution, i.e. more details are visible on the image.



Resolution is measured in various units. Typical units are micron (1 micron corresponds to a pixel width of 1 micrometer), ppi / dpi (pixels per inch / dots per inch) or in the metric system pixels per millimeter.



Higher resolutions yield increased precision. However, the increase in precision is marginal once a certain threshold resolution is exceeded.



On the other hand, every doubling of the resolution results in a fourfold increase of the image file size. Scanning takes more time at higher resolutions, files take up more disc space, and image analysis requires more memory and time.

Image resolution is part of the parameters that can be adapted in the imaging software.

The following table shows the relation between resolution, spot sizes and file sizes for some typical resolutions.

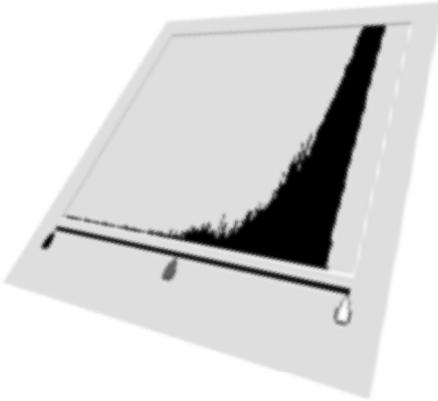
image resolution [dots per inch]	pixel width [inch]	image resolution [micron]	diameter [pixel] of a 1mm-diameter-spot	area [pixel] of a circular 1mm-diameter-spot	diameter [pixel] of a 5mm-diameter-spot	area [pixel] of a circular 5mm-diameter-spot	width of a 20cm gel image [pixel]	file size [MB] 8 bit grey levels	file size [MB] 16 bit grey levels
100	0.0100	254	4	12	20	304	787	0.6	1.2
150	0.0067	169	6	27	30	685	1181	1.3	2.7
200	0.0050	127	8	49	39	1217	1575	2.4	4.7
300	0.0033	85	12	110	59	2739	2362	5.3	10.6
400	0.0025	64	16	195	79	4869	3150	9.5	18.9

Typical image resolutions and corresponding spot and file sizes.

For image analysis of protein spots, the smallest spots should have a minimum diameter of about 5 to 10 pixels. As an example, with a resolution of 200 dpi a very small spot with 1 mm in diameter will have a diameter of about 8 pixels in the resulting image.

Increasing the resolution after imaging does not add information to the image, while reducing resolution entails loss of data. However, this loss is acceptable provided that the diameter of the smallest spot of interest does not drop below 5 pixels.

Dynamic Range



Enlarging the distance between signal and background is one of the major tasks in image generation. Maximizing the distance between background and top intensity in an image means to fully exploit the dynamic range signal recognition. The more signal intensity levels are recorded in the image the more accurate is the quantitative analysis.

There are several ways to increase the dynamic range:

Cutoff high background by a preset. In the initial imaging step the user can usually specify the lowest and highest intensity signals of interest. The measured signal intensities below or above this range of interest are set to zero or to the maximum, respectively, while signal intensities within this range of interest are evenly distributed between zero and the maximum for the bit depth. This makes sense if the A/D converter can discriminate more intensity levels than can be stored in the image file. An alternative approach is transforming the data from the A/D converter by using gradation / calibration curves (see also section "Data Reduction and Image Calibration").

Controlling the conversion from color to grayscale. Most imaging devices create grayscale images by converting a color image to a grayscale image file based on average intensities of the different color channels. You can improve the resulting image in some situations by controlling the color-to-grayscale conversion yourself, e.g. by extracting the color channels with high absorption or (even better) with the highest dynamic range:

- An image of a Coomassie blue stained gel can be improved by excluding the blue color channel from the color image before converting it to a grayscale image.
- A similar approach can be used for the brownish silver stains. They most efficiently absorb blue light, so extracting the blue color channel (and excluding red and green) gives the best results.

Increasing the voltage of the light detector. The voltage (sensitivity) of the light detector may be increased if the intensity range is not completely covered by signal intensities in a fluorescence scan. This not only increases the signals overall, but also the absolute distance between background signal and strongest intensity signal on the gel image.

Please be aware of saturation effects! (See also section "Saturation Effects".)

Increasing the exposure time. Similarly, the exposure time may be prolonged if the intensity range is not completely spanned by signal intensities in a camera system. As for the approach above, this increases both the overall signal intensities and the absolute distance between background signal and strongest intensity signal on the gel image.

Please be aware of saturation effects! (See also section "Saturation Effects".)

Light Intensity and Detector Sensitivity



The intensity of the light source directly influences differentiation of high density spots. Especially for **fluorescence imaging** high performance light sources are absolutely necessary. In this field of imaging normally lasers or UV lamps are used. Lasers deliver highly concentrated light and can effectively excite fluorescent dyes. Depending on the fluorescent dyes different lasers with specific colors as well as excitation and emission filters are needed. Usually **lamps and lasers need a warm-up phase** to reach their highest performance.

The light harvesting detector and the A/D converter collect the light that has passed through or that is emitted from the gel and translate this information into computer readable signals: The detector (CCD element or PMT) converts the light intensity to an electrical signal that is subsequently digitized by the A/D (analog to digital) converter. These data are stored in the image file.

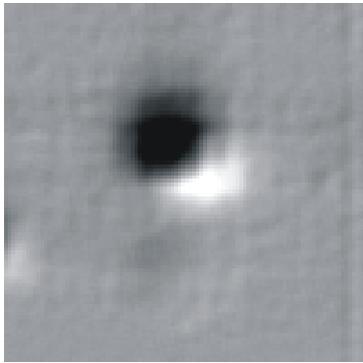
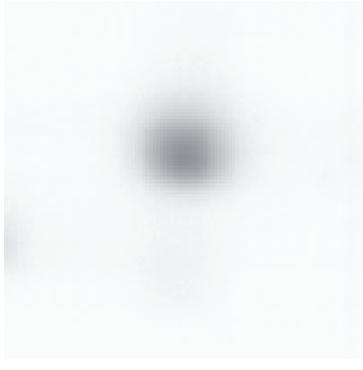
Scanners. It is common to use office document scanners to detect absorbing dyes (Coomassie, silver, X-ray film). The manufacturers usually claim a differentiation of gray levels up to an optical density (OD) of 2.0. This is mostly sufficient for gel documentation purposes. However, it is insufficient for differentiation of high density spots reaching optical densities near 3.7 to 4.0.

Document scanners resolving ODs up to 3.7 or 4.0 can be found in the premium or professional product lines of well known manufacturers. **Warming up the lamp** before starting the scan process is highly important because the lamps become more powerful when they reach their working temperature. Extensive usage of the scanner causes aging of the light source resulting in weaker light intensity.

In many high performance scanners for fluorescence detection also very weak signals can be detected. In these scanners the voltage of the light detector (PMT - photo multiplier tube) can be adapted. Higher voltages increase the sensitivity for very weak signals but may also increase image background. Unfortunately increasing PMT voltage also results in amplifying noise. Therefore PMT voltage should be modified with care. Another pitfall is simultaneous enhancement of strong signals "boosting" very intense spots beyond the saturation threshold.

Cameras. Camera systems usually cannot be adjusted to changing light intensity. The system sensitivity can be modified by varying the exposure time, similarly to changing PMT voltage for a scanner. Because the construction of cameras is different from scanners, homogenous illumination of the complete gel is an important quality aspect. Some camera systems can compensate for an inhomogenous illumination, which has to be adjusted frequently.

Saturation Effects



A perfect spot without any saturation effect.

Saturation effects heavily affect quantitative data because they virtually truncate the peaks of high intensity spots. These truncations distort the quantities of spots. Saturation effects may occur for several reasons:

Intense staining can cause complete absorption of light passing through the spot.

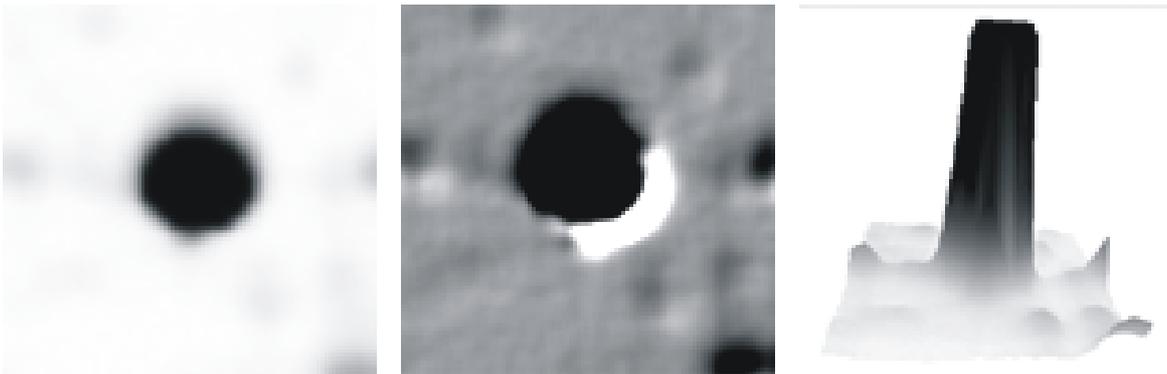
Dyes can appear in very high concentration. Some staining techniques accumulate pigment on top of high intensity spots losing the linear amount-to-intensity correlation. This can be observed in all techniques based on the photographic silver nitrate staining (silver staining, X-ray film).

Avoid saturation effects in the lab by

- reducing staining or increasing destaining time, or
- loading less protein on the gel.

Heavily fluorescing gels may overload the light detector, or intensively excited light detectors may overload the A/D converter, which can no longer differentiate between strong signals. Similarly, if a gel is overexposed to a phosphorscreen during phosphorimaging, the capacity of the screen to accumulate energy from radioactive decay events may be exhausted.

Saturation effects usually have a planar appearance. Many imaging device software packages highlight saturated image regions. In the 3D view of the gel image they can also be observed.

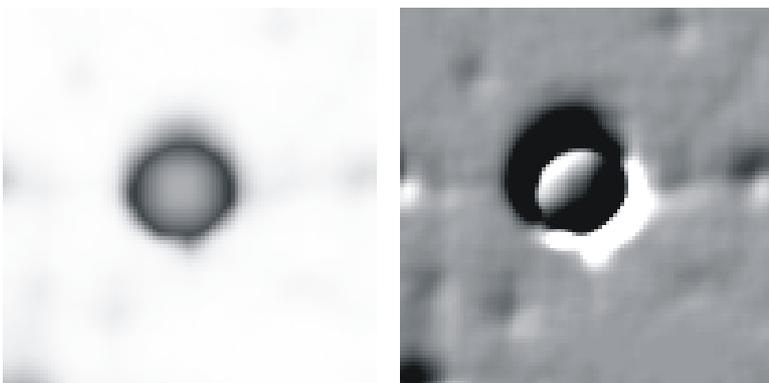


A saturated protein spot.

To reduce or eliminate saturation effects, repeat the imaging and

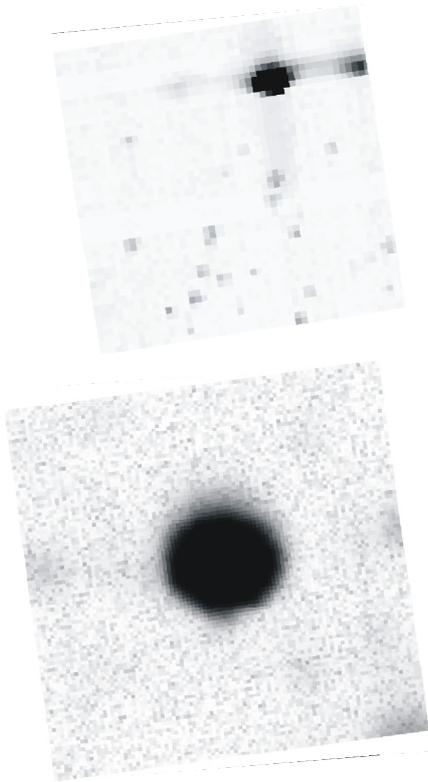
- increase light intensity (not for fluorescence staining),
- reduce the PMT voltage (scanner), and/or
- reduce the exposure time (camera, phosphorscreen).

For classical silver staining techniques saturated spots generally appear as typical "doughnut spots". Silver staining should not be used in quantitative proteome analysis because the relation between silver stain intensity and protein amount is usually not linear.



Silver stain: A "doughnut protein spot" with strongly diminished central staining intensity.

Artifacts: Improve Device Handling



Avoiding background, artifacts and noise is essential. They may interfere with the spot detection process: Gel breaking separates spots, sprinkles may mislead the spot detection, noise can cover low intensity spots etc. Artifacts also affect the quantitation process. The weaker the signals are the stronger is the effect of artifacts on quantitative analysis. Very low signals may be observed due to faint spots, very low sample amounts or poor incorporation of radioisotopes. Frequently, it is better and less frustrating to repeat the experiment instead of performing analysis of suboptimal images.

Background may be caused by insufficient destaining, fluorescing glass plates, gel coverings and backings. Low fluorescence equipment minimizes such problems. Also inappropriate selection of optical filters for fluorescence imaging may cause background. Reevaluate which light sources and filters are suitable for your analysis.

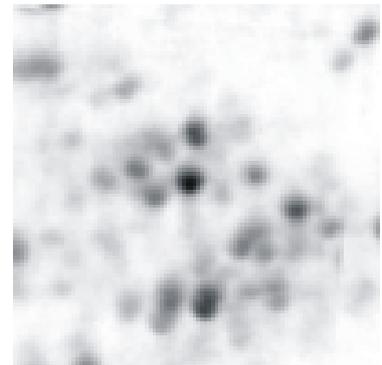
Noise can be produced by high PMT voltages enhancing also random signals. Adapting the PMT voltage solves this problem. Similarly, phosphor screens that have not been used for a longer time accumulate noise. Erasing the screen immediately before exposure solves this problem. Try to optimize the imaging procedure and avoid downstream manipulation of your images.

- Exclude regions containing artifacts during imaging
- Image all gel images using the same orientation

A common trick is to cut off one corner of the gel. Cut off the low Mw, low pI corner. Make sure that this corner becomes the lower left corner in the image for every gel you scan. This has the added advantage that your images will have the orientation that is usually required by the proteomics journals (pI increasing from left to right, Mw decreasing from top to bottom). If you need to rotate or flip the image we recommend using the software that came with your imaging device. General purpose image processing software (e.g. Adobe Photoshop) may not preserve the vendor specific grayscale calibration information in the image file.

- Ghost images or spot shadows

Do not move the gels during exposure or scan. Be aware of device vibrations during the imaging process. Fix the gel on the device surface (use suction cups).



Ghost images are problematic because they are hard to recognize.

- Horizontal or vertical lines

Sometimes single CCD elements in CCD bars of a desktop scanner fail to work. The corresponding line of the image remains empty. The only way to solve this problem is replacing the CCD element or the whole scanner.

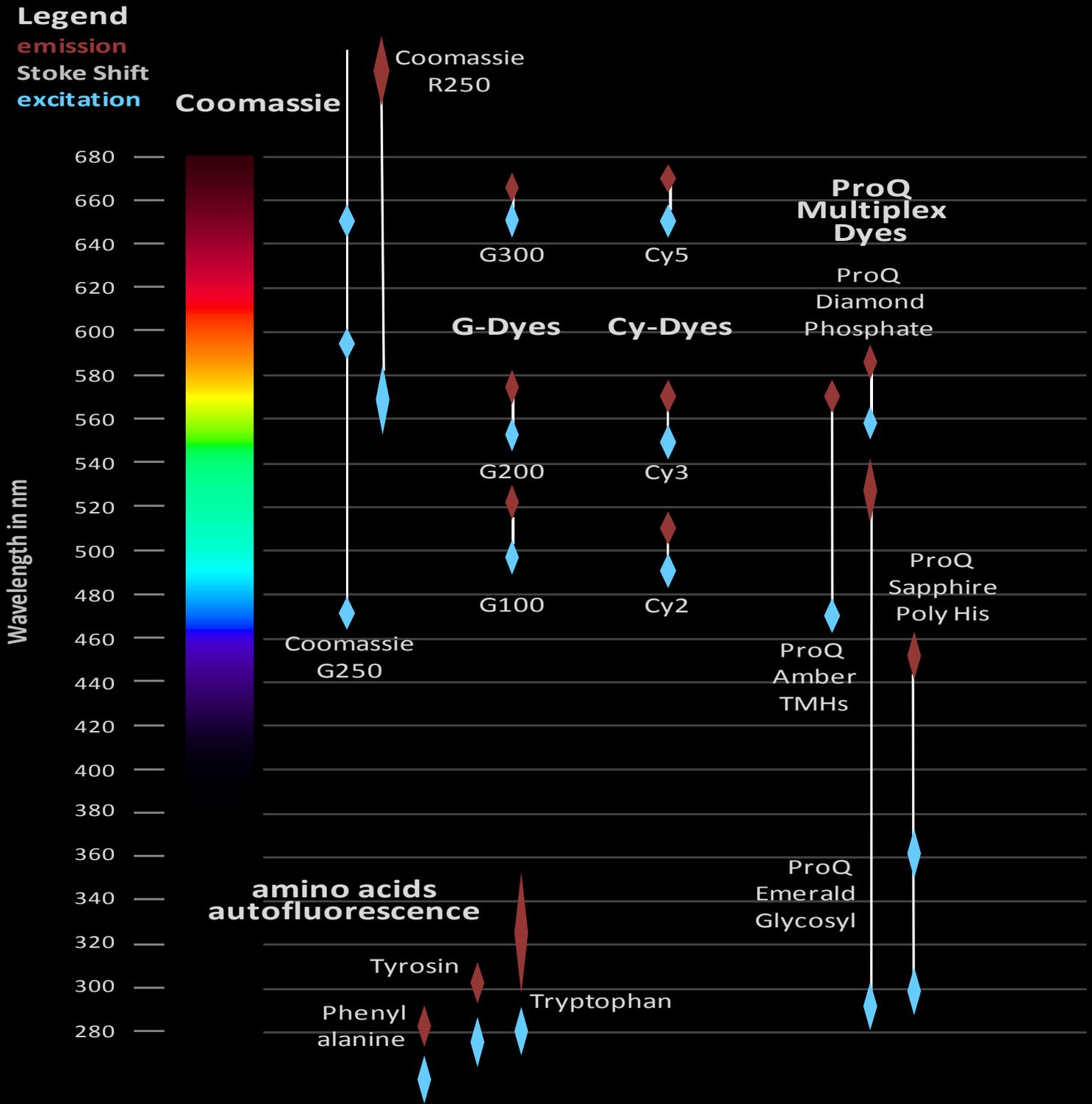
- Artifacts or noise from phosphorscreens

Decontaminate or replace phosphorscreens; scan auto-exposed phosphorscreens before use to locate and remove contaminations. Erase the screen before exposure to eliminate accumulated noise.

- Artifacts and scratches on glass panels

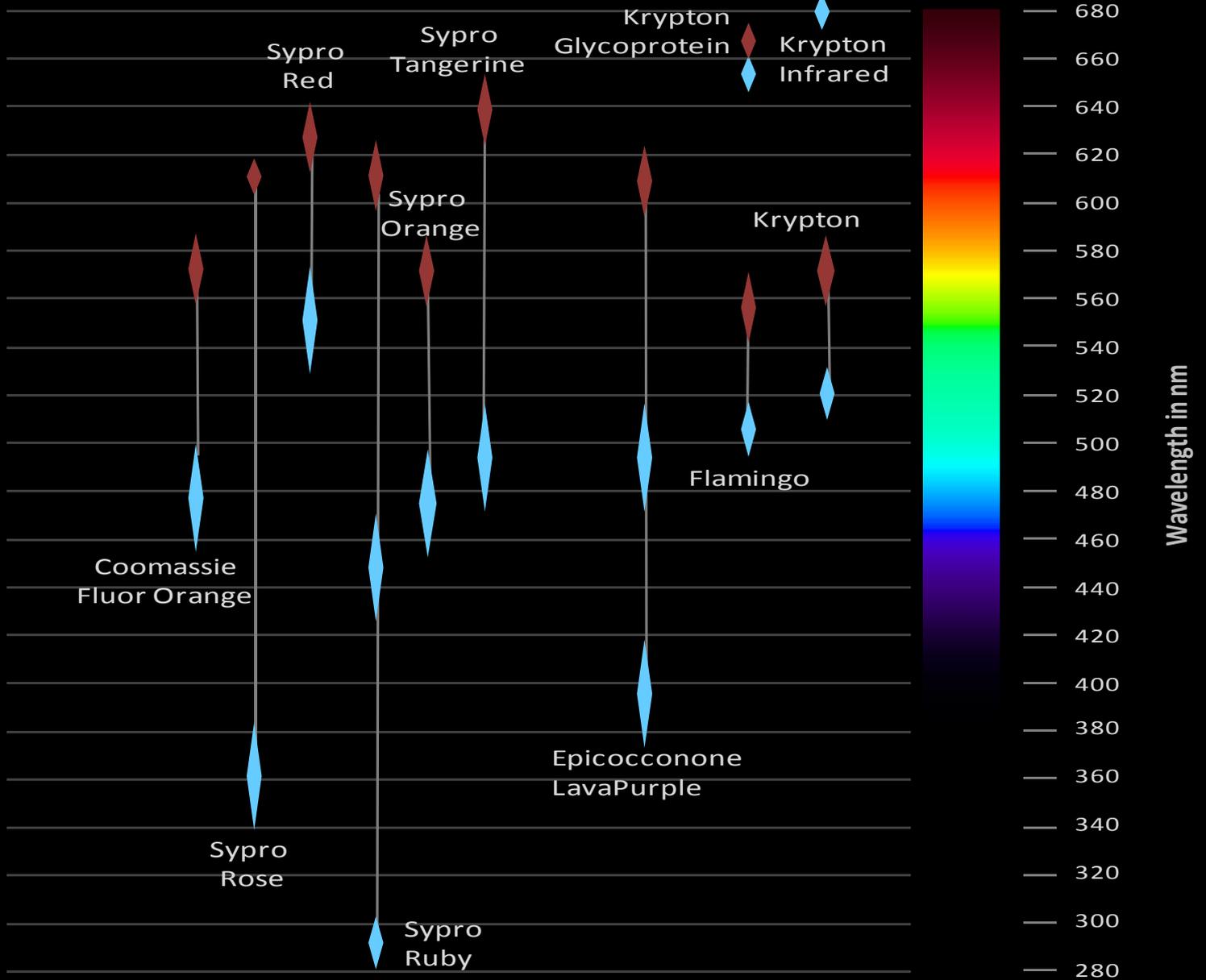
Handle your imaging devices with care and only use a mild detergent and wood free tissue (do not use recycling tissue!) for cleaning. Keep all glass panels clean and free of dust and fat.

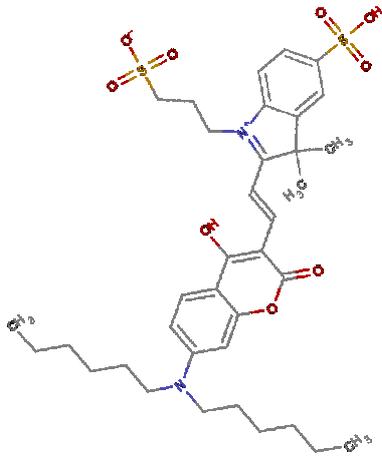
Excitation / Emission of Fluorescent Dyes



Single Channel fluorescent Dyes

Legend
■ emission
■ Stoke Shift
■ excitation





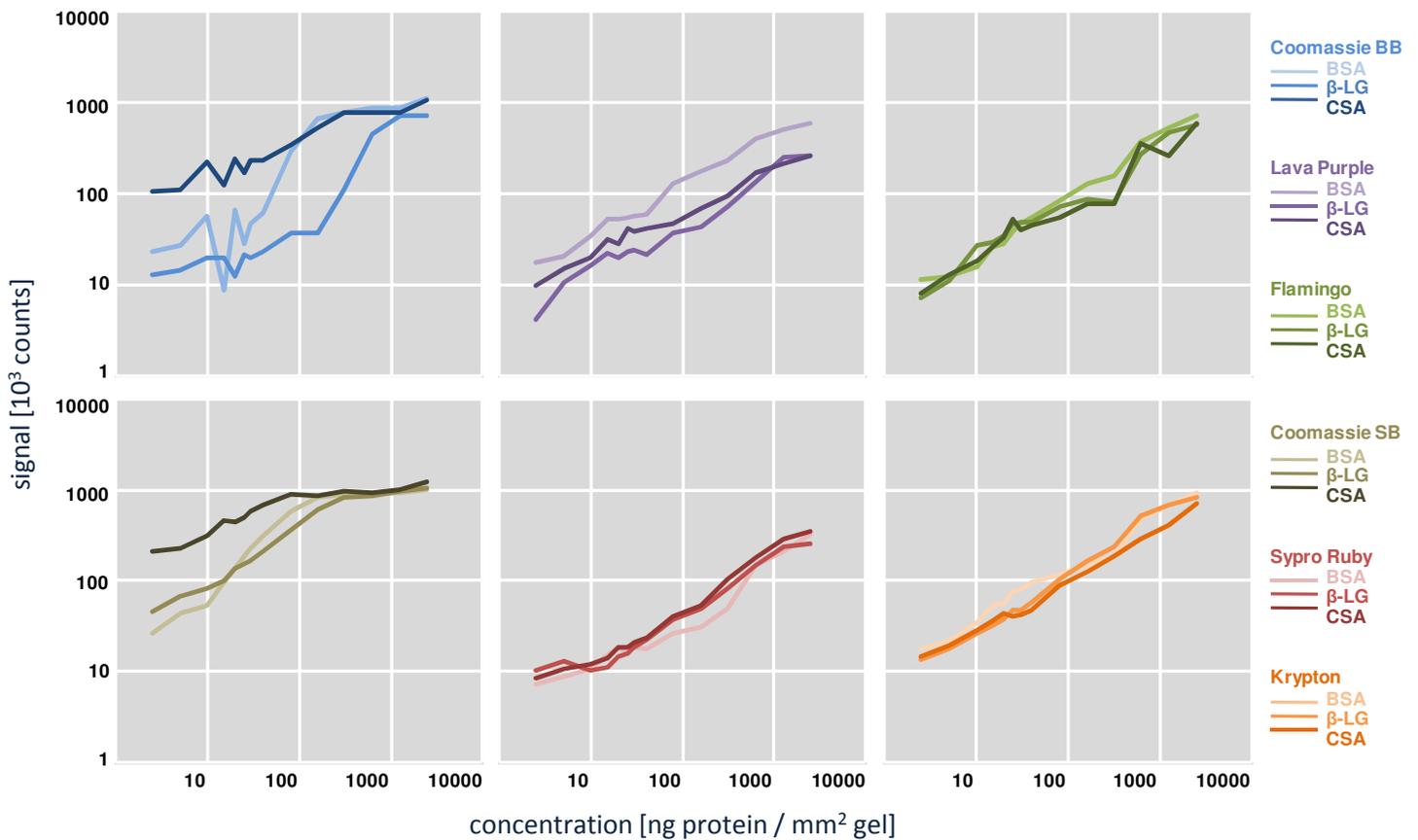
Appropriate Protein Dyes

Currently various protein dyes are available. In general two types can be distinguished: covalently binding dyes (the dye chemically binds to the protein before electrophoretic separation – e.g. G-Dyes, Cy-Dyes, etc.) and non covalently binding dyes (the dye binds electrostatically to proteins after electrophoresis).

Besides low protocol complexity and attractive price the following aspects should be considered when choosing a protein dye:

- Is the dye suitable for the protein concentration range of interest, the expected sensitivity and dynamic range?
- Does the dye tend to show artifacts (sprinkles, saturation effects, noise, background)?
- Has any significant protein-to-protein variability been reported for the dye?
- Does the dye match the characteristics of the available imaging system, e.g. in terms of excitation and emission wavelengths?
- Is the staining protocol for the dye tolerant against minor handling deviations to avoid unacceptable variances of resulting signal intensities?

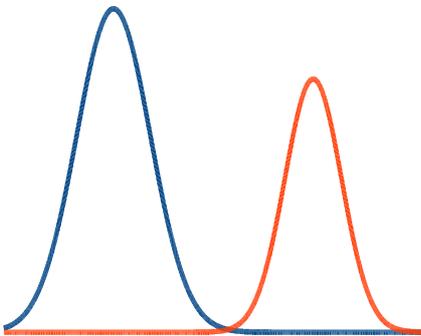
The following figure depicts the dynamic ranges, sensitivity and protein-to-protein variation of different post-electrophoretically applied dyes.



Dye specific signal intensities for different proteins and concentrations¹.

1 – Varying amounts of proteins (bovine serum albumin, beta-lactoglobulin, chicken serum albumin) were stained with the indicated dyes in a scientific study of the proteomic group of the Institute of Microbiology – Greifswald University (Kuzinski, Greifswald, 2007).

Detection of Fluorescent Dyes and Multifluorescence Setups



Light Source. Each fluorescent dye absorbs light of its specific excitation wavelength and emits light of lower energy, i.e. of a longer wavelength. The intensity of the emitted signal is maximized if the light from the imaging device exactly corresponds to the excitation maximum of the fluorescent dye.

Because common laser scanners contain lasers of fixed wavelengths (red, green, blue – in some devices also violet, blue-green or infrared), the combination of laser and dye (excitation maximum) is always a compromise. Devices using light sources with a continuous spectrum provide more flexibility if a limited range of the spectrum can be selected for excitation. The light intensity of the spectral band of interest unfortunately is much weaker than that of laser scanners.

Emission Filters. Optical long pass filters (LP filters) are used to detect as much of the emitted light as possible. LP filters are specified by the wavelength above which light can pass the filter. The more light of the emission spectrum passes the filter, the higher are the sensitivity and dynamic range of signal detection. Thus in all experimental setups with only one dye an LP filter slightly above the excitation wavelength should be employed.

In multiplex designs (like the DIGE approach using G-dyes or Cy-dyes) it is absolutely mandatory to avoid detecting a mixture of light being emitted by different dyes. This can already happen even if only one dye is excited, because the light emitted by this dye might excite one of the other dyes causing secondary fluorescence. When using an LP filter all emitted light would be detected, regardless of whether it originates from the dye of interest or the other one(s). Such unwanted secondary fluorescence is blocked by using band pass (BP) filters.

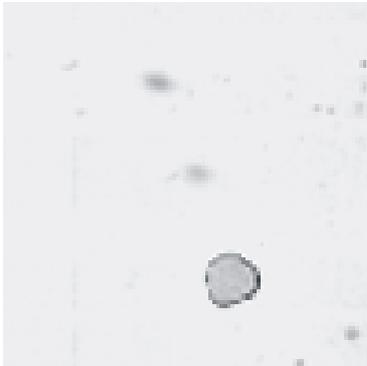
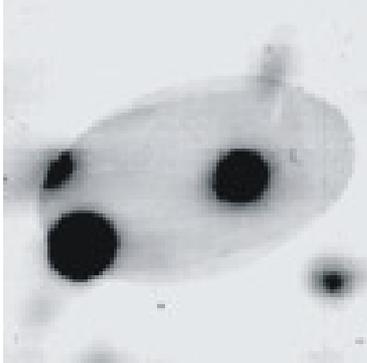
BP filters are specified by the wavelength at which light transmission is highest or wavelength range for which the filter is transmissive. For instance, an optical 500/20 BP filter is most transmissive at 500 nm, but also light in the range from 490 to 510 nm can pass. High quality BP filters are almost equally translucent across the whole specific band width. Narrow band filters may exactly confine the detection (emission) wavelength; however, for dyes with broad emission bands appropriate broad band pass filters enhance the detection sensitivity.

To summarize, the selections of dyes and the imaging device are interdependent. The light source needs to be compatible with the excitation wavelengths of the dyes. Furthermore a filter set should be used that permits separate detection of the specific light signals emitted by each dye.

Imaging time. Multiple scanning of multiplex gels is rather time-consuming (up to 20 minutes per scan in established systems) and may result in gel shrinkage owing to evaporation of gel buffer or water. Consequently spot positions may be shifted, making it difficult to match spots during the image analysis. This can be avoided by keeping the scan surface wet enough and/or by using Delta2D's innovative approaches including image warping and creating complete expression profiles¹. Furthermore, camera systems require a much shorter exposure time.

1 - <http://www.delta2d.com>

Artifacts: Improve Gel Handling



Bubbles may interfere with quantitation.

Several artifacts can be avoided by appropriate gel handling:

- Air bubbles and water droplets

Use enough water between glass panel and gel but be aware of water seeping into your imaging device. Silicone sealants may help to close problematic grooves.

- Newtonian rings

Try to avoid them using Kapton® tape or glass plates. If this is not possible focus the laser beam inside the gel.

- Artifacts on the gel surface

Try to focus the laser beam inside the gel if your imaging device supports this feature.

- Fingerprints on the gel

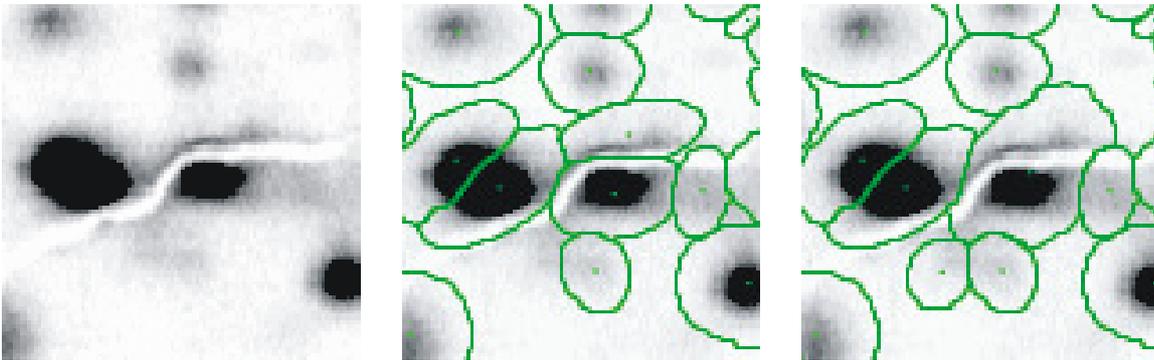
Use (powderfree) gloves and/or clean the glass panels of your imaging device

- Labels on the gel

Using permanent markers for gel labeling sometimes interferes with the scan. To simplify the subsequent analysis please make sure that the label does not overlap with parts of the gel or spot pattern.

- Gel ruptures and gel pieces

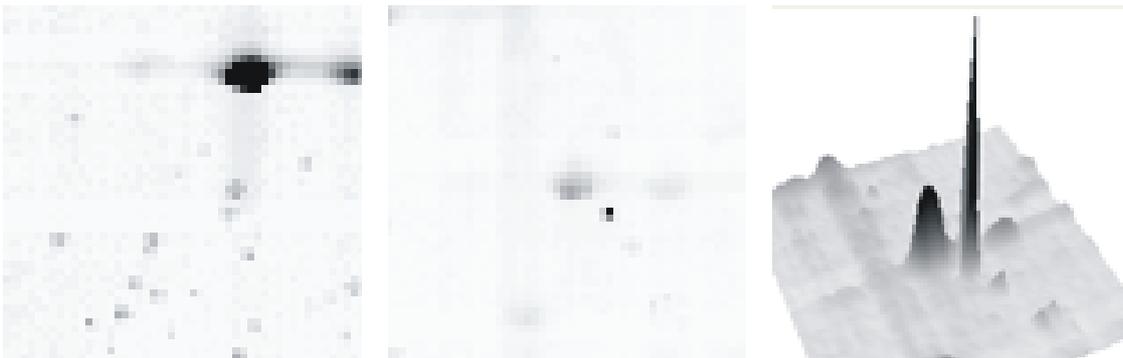
Work carefully and use gel strengthener to give the gel more stability. If necessary try to assemble the gel pieces to minimize the distance between them before imaging.



Spot ruptures cause problems with spot detection. This may be corrected by manual spot editing.

- Fluorescent sprinkles

Rinse the gel with destainer or water and avoid evaporation. Use powder free gloves, dust free experimental equipment in a dust free environment.



Fluorescent sprinkles can disturb the spot detection.

Bit Depth

Signal intensities are encoded as numbers in the image file. In grayscale images this is one number for each pixel, while in color images one number for each color channel has to be stored per pixel (e.g. in RGB color space one intensity value for red, green, and blue).

Make sure you use grayscale instead of color images: the extra color information does not provide benefit for the subsequent analysis of 2D gels.

When your image file is, for example, a "16-bit TIFF" file this means that each number is encoded with 16 bits, i.e. represents one of 65,536 ($= 2^{16}$) different intensity levels. In contrast, an 8-bit image file only includes 256 ($= 2^8$) different intensity levels. The number of bits per color is also called the bit depth (or color depth).

bit depth	intensity levels	example
1 bit	2	black and white FAX image
8 bit	256	GIF image
12 bit	4,096	TIFF image
16 bit	65,536	TIFF image

Typical bit depths.

TIFF images can have different bit depths. This is one of the reasons why TIFF is a widely used image file format.

Generally, higher bit depth is better for quantitative image analysis. A tradeoff exists between accuracy and storage space: a 16 bit TIFF file two times larger as the respective 8-bit file, but comprises 256 times more intensity levels. Many common image processing programs are not able to deal with bit depths above 8 bit.

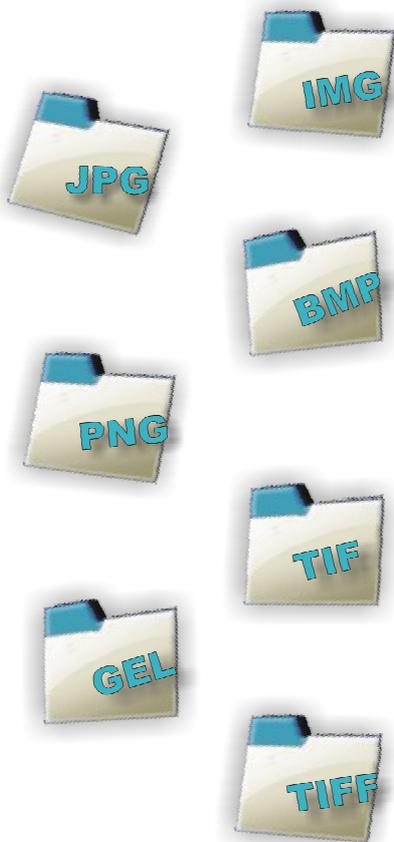
Decreasing bit depth in an existing image file normally results in loss of accuracy. Conversely, it is not recommended to increase bit depth after the imaging process: transforming an 8-bit image to a 16-bit image will result in again 256 out of 65,536 possible gray values.

You can find more information about bit depth in image file formats in the Wikipedia Category: [Color Depth](#)¹.

Although high bit depth enhances image analysis, particularly protein spot quantitation, it is often not visible on the screen or in print due to restrictions of the respective technologies.

1 - http://en.wikipedia.org/wiki/Color_depth

Image File Formats: Image Compression



Compression of image data is achieved with algorithms that reduce the size of a gel image file while retaining all or most of the image information. If you use calibrated image file formats, image compression is not an option, because compressed file formats do not store the calibration information.

Image compression algorithms can be classified as lossy or lossless methods. Lossy compression means that information can be lost in the image details, yielding a higher compression ratio and smaller file sizes than lossless methods. Uncalibrated files may be saved in file formats that use lossless data compression, e.g. subformats of TIFF / TIF or PNG. Often compression to 50 % of the original size is possible. The commonly used JPG format uses a lossy compression method. Large compression ratios may heavily change or destroy your data. Even for low compression ratios, there is still an unknown influence of compression on spot quantities.

We recommend avoiding the JPG file format for image analysis purposes.

Image compression only saves disc space but does not affect the amount of working memory (RAM) needed for image analysis because compressed files are also completely extracted before starting the analysis.

The following table summarizes the properties for commonly used image file formats.

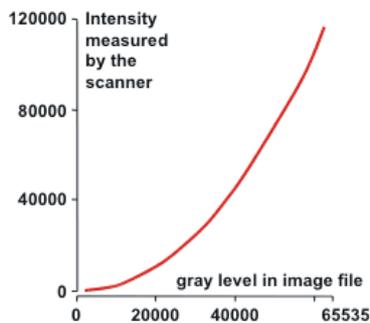
format	compression	gray levels	use for quantitative analysis	calibration
GIF	lossless	256	no	no
BMP	no	2; 256	(yes)	no
PNG	lossless	256	yes	no
JPG	lossy	256	no	no
TIF(F)	no (lossless)	2;256;1024;4096;65536	yes	no
GEL	no	1024;4096;65536	yes	yes
IMG/INF	no	1024;4096;65536	yes	yes

Commonly used image formats. Avoid formats like JPG.

You can find more information about image file formats in the Wikipedia Category: [Image File Formats](#)¹

1 - http://en.wikipedia.org/wiki/Image_file_formats

Data Reduction and Image Calibration



Example of an image calibration curve.

Some imaging devices can measure more intensity values than fit into the available image formats. One way to deal with this is to convert the signal intensities linearly into the bit depth offered by the image file format. Example: The Analog to Digital (A/D) signal converter can deliver 1,024 different intensity levels, but the image file format is limited to 256 levels (8-bit depth). Linear transformation condenses four A/D converter levels to one intensity level in the image. Obviously, this condensation process causes loss of accuracy.

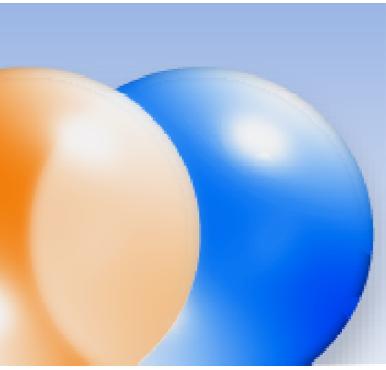
Especially if images are captured with light scanners condensation can be improved by using only the real dynamic range of the measured signals as delivered by the A/D converter. Example: The A/D converter can deliver 1,024 different intensity levels. If the gel scanning only renders intensities between 128 and 920, only these 792 intensity levels have to be encoded in the image file: thus three A/D converter levels are condensed to one image intensity level. Compared to the example above, accuracy is increased by 25%.

There are instruments that can measure 100,000 and more different signal intensity levels, far more than can be encoded in a TIFF or similar file format. In order to save as much information as possible in these files, measured intensities are transformed to pixel gray values according to a nonlinear calibration curve during scanning. Typically, the respective nonlinear calibration curve ensures that lower intensities are encoded with higher accuracy than higher intensities.

Calibration curves are vendor specific and not saved in standard image file formats like TIFF. For this reason vendor specific formats have been created like GEL or IMG/INF which include specific parameters of the calibration curve.

On the basis of the calibration curve the image analysis software decodes the originally measured signal intensities. A wrong or no calibration curve results in incorrect quantitative values. If your image analysis software cannot interpret the vendor specific file format, make sure that you save your images in a standard image file format like TIFF.

Commonly used image processing packages (e.g. Photoshop) ignore grayscale calibration. It is even possible that calibration information is lost when saving images with these packages.



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