# APPROPRIATE IMAGE PROCESSING

## – Explanation of Submission Guidelines of Journals –

Japan Agency for Medical Research and Development

Image processing of biological images is a relatively new field of study. In particular, there are extremely few image processing textbooks for biologists, and hardly any opportunities for them to acquire systematic knowledge or to learn techniques of this. This booklet therefore sets out to explain appropriate image processing of biological images based on the submission guidelines of journals.

As the submission guidelines will change with time, it is necessary to check for updates. In addition, after the paper has been published in the journal, you must not only securely retain the images and data that are directly linked to the paper, but also all the original images and other raw data for at least the period specified by their research institutions.

# 1. Image processing guidelines documented in scientific journals

The techniques of image processing of biological images often completely vary by research purpose. As there are many image processing techniques for various types of biological images, it is extremely difficult to define a process in which "such image processing technique(s) are considered as image fraud". For this, scientific journals explicitly state the minimum standards in the submission guidelines. While the submission guidelines for all scientific journals are not exactly the same, it can be said that the major scientific journals' rules regarding image processing are in line with each other.

In 2004, the Journal of Cell Biology (JCB), which publishes many papers in the field of cell biology, was the first to set image processing guidelines ahead of other journals[1]. The contents of the current submission guidelines of JCB or other journals under the Nature Publishing Group do not deviate much from the image processing guidelines that was published in 2004. Put it differently, the journals have only set the minimum requirements to be strictly followed, since image processing serves many different purposes.

The objective of this booklet is to explain the image processing guidelines set by the Nature Publishing Group by taking account of their maintenance, accessibility, and influence. As mentioned above, since most of these guidelines are common to other scientific journals as well, you should also strictly follow them when submitting your manuscripts to scientific journals other than Nature.

## 1.1 Submission guidelines of the Nature Publishing Group

Nature's submission guidelines are published online and can be accessed from the URL below.

https://www.nature.com/nature-research/editorial-policies/image-integrity

It outlines the guidelines for biological images in general as well as for electrophoretic gel images and micrographs as follows.

**For biological images in general**

- Save the original images; submit them at the request of peer review.
- Submit high-resolution color images of 300 dpi or higher.
- Keep image processing at a minimum; submit images that represent the original images.
- Specify the software as well as the image processing technique(s) used.
- Avoid unnecessary image composites. If needed, add a white or black line to demarcate one image from another.
- Do not use touch-up tools.
- Apply the same image processing across the entire image.
- Apply the same image processing for comparable images.

**For gel images**

- It is recommendable to combine gel images to improve visibility but necessary to specify it; submit the original gel images when requested.
- Do not directly compare bands from different gels; specify if you do so; run molecular markers and controls on the same gel.
- Leave enough margins around the cropped band.
- Avoid excessive contrast enhancement. Submit the original images as supplementary information if the contrast has been excessively enhanced.
- Avoid applying non-linear gray level transformations into quantitative evaluation.

**For micrographs**

- Submit the original images when requested.
- Apply image processing across the entire image.
- Avoid excessive contrast enhancement.
- Specify when applying pseudo-coloring and/or non-linear luminance transformation.
- Specify when adjusting individual color channels in color images.
- Specify the type of microscope, lens, and software used.
- When displaying a heatmap using pseudo colors, specify the information corresponding to each color.
- Specify the image processing technique(s) used.
- When changing the resolution of the image, specify it with the technique(s) used.

## 1.2 Submission guidelines of journals other than the Nature Publishing Group

On top of Nature, there are other scientific journals developing pioneering initiatives. Some notable examples are JCB and EMBO Press.

**JCB: JCB Data Viewer**

JCB, who has been developing pioneer initiatives on image handling, recommends authors to upload the original images and document about the image processing technique(s) used in their JCB publications in their database, the JCB Data Viewer[2)]. Readers can apply image processing on the original images and edit the published images while referencing the papers published in JCB.

**EMBO Press: Data Integrity Analyst**

EMBO Press has their own team of dedicated data integrity analysts that manually check all figures and images submitted to EMBO journal. It was reported that approximately 20% of the checked images were found to have flaws, with about 1% of them falling under the category of serious image fraud[3)].

While each scientific journal has set their own guidelines regarding image processing, these guidelines have much in common and you would be able to avoid being suspected of committing image fraud if satisfying the following minimum requirements. The next section will go into detail on the image processing techniques that are deemed appropriate.

# 2. Appropriate image processing

Even if mistakes are not malicious, the career of the researcher would be damaged once suspected image fraud. If you retain the original images and documents the image processing techniques, you are able to defend your claim that the figures created correctly represent the original images. On the other hand, if these data are not saved, you may have to exhaust much time to dispute the allegations.

It is therefore important to save the original images as separate files when processing images as well as to document the image processing techniques used.

Also, if suspicions of image fraud in scientific publications are raised, the problem is almost always with either the contrast adjustment or the composition of images.

In this section, “Retaining original images & documenting image processing techniques”, “Contrast adjustment without beautification”, and “Examples of spliced gel images” are explained.

## 2.1 Retaining original images & documenting image processing techniques

You might be asked to submit original images not only when allegations or suspicions of image fraud arise, but also during the peer review process. Since most of the functions of image processing software are irreversible, you may find yourself in trouble if the original images are overwritten by the edited ones. To avoid such a situation, you need to save the original images as separate files while editing.

It is also important to document all the image processing techniques and the parameter settings. According to the submission guidelines of scientific journals, authors are required to at least explicitly state the “name of software used for image processing”, “software version”, and the “main image processing techniques and parameter settings”.

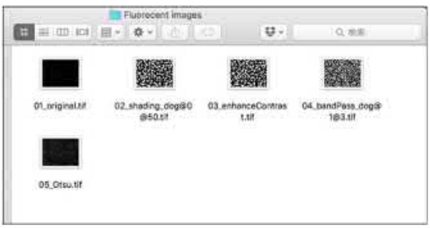



Figure 1-1: It is important to save the original image while applying image processing. Also, in the above group of images, the image processing techniques and the parameter settings are saved in the file names.

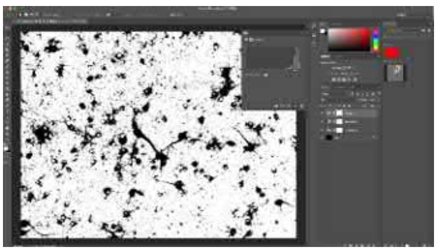


Figure 1-2: In the case of Photoshop, it is possible to apply image processing on an additional layer over the original image.

## 2.2 Contrast enhancement without beautification

It is rather recommended to enhance the contrast of biological images to improve visibility of the images. However, excessive contrast enhancement may turn out to beautification (blocked-up shadows or blown-out highlights). This would result in blocked-up shadows (where the parts turn completely black) and blown-out highlights (where the parts turn completely white); both are referred to as "beautification". With blocked-up shadows or blown-out highlights, it is not possible to determine whether the result is purely that of contrast enhancement, or author is trying to hide unfavorable data. This would invariably lead to suspicions of image fraud.

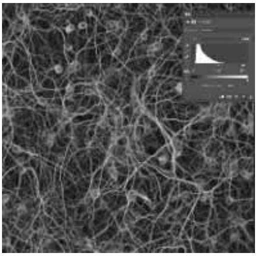


Figure 2-1: When adjusting the contrast of an image, it is possible to avoid beautification by adjusting the brightness histogram on both ends.

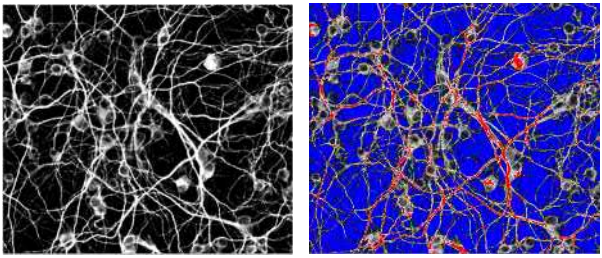


Figure 2-2: An example with excessive contrast adjustment. Blocked-up shadows (blue) and blown-out highlights (red) are observed.

### 2.2.1 Appropriate contrast adjustment for a single still image

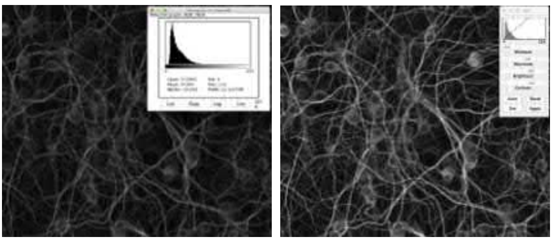
It is recommended to adjust the contrast of images by any of the following three techniques.

1. Make level adjustments using both ends (minimum and maximum target signals) of the brightness histogram.
2. Adjust the contrast to induce 1-2% of overexposure and underexposure in the image.
3. Adjust the contrast such that the background brightness is visible.

It is possible to edit the image to get a clean one while avoiding excessive beautification by using these techniques.

In general, the first technique that make level adjustments using the minimum and maximum target signals is the best. This allows you to achieve an image with visible background brightness but with no blocked-up shadows (underexposure) or blown-out highlights (overexposure) (Figure 2-3).

The second technique is effective where images are required to be clean-cut (especially for images for the covers of scientific journals or images for commentary articles for the general public) while ensuring the quality of the data for scientific journals (Figure 2-4).



Original image

Contrast-adjusted image

Figure 2-3: An example of level adjustments using both ends (minimum and maximum target signals) of the brightness histogram

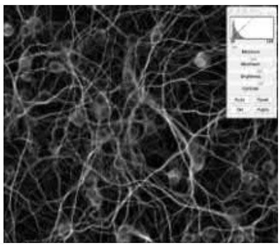


Figure 2-4: An example of an image where the contrast was deliberately adjusted to induce 1-2% of underexposure and overexposure

The third technique makes it easier to quantitively visualize the signal region of interest. In fact, the human eye perceives both completely white and black areas almost equally bright; unlike numerical data, the ability to quantify senses is thus lost. In order to quantitatively visualize images, it is critical to contain the signal region of interest in the gray region (for the human eye to quantitatively visualize as well). This would also guarantee the quantitativity even when the papers or images are printed.

Figure 2-5: The above image has a width of 256 pixels, with a gradation in the brightness spectrum from 0 (completely black) to 255 (completely white). However, the quantitativeness of the extreme ends of this spectrum is lost to the human eye (and print outs).

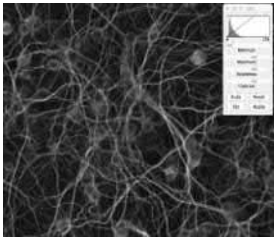


Figure 2-6: An example where the contrast is adjusted such that the signal region of interest is quantitatively visible.

### 2.2.2 Adjusting contrast of multiple images for comparison

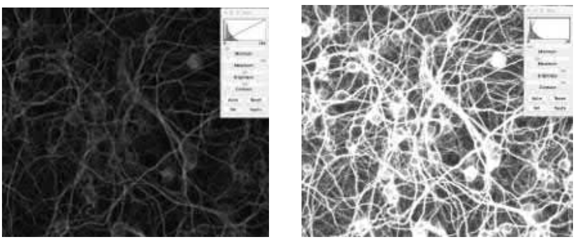
In the above section, techniques how to adjust the contrast of a still image are described. However, if there are multiple biological images to be juxtaposed beside the target images for comparison, it is necessary to apply the same contrast adjustment setting across all the images.

Take for example fluorescent micrographs: even if the minimum brightness values (background brightness of images) are the same, the maximum brightness values of the signals (fluorescent substances and fluorescent proteins) would vary by image. When quantitatively evaluating the fluorescence brightness across multiple images, you should consider the maximum brightness values of all images as the upper limit when adjusting the levels. Alternatively, you could also adjust the levels to the lower limit (minimum brightness value) only.

### 2.2.3 For images visible only under high contrast

In an ideal situation, only the area or region of interest would be beautifully imaged. However, there are times when other areas are imaged brighter because of autofluorescence or non-specific fluorescence. If the contrast of the image is adjusted according to the area of interest, the surrounding areas would be excessively beautified instead.

In such cases, you could consider adjusting the contrast gradually, or submit the original images as supplemental figures.



Original image　　　　Contrast-enhanced image

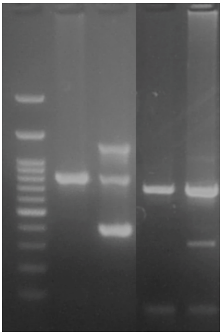
Figure 2-7: An example where the area of interest exhibited poor signals.
One could either display images where the contrast was adjusted gradually or submits the original image in supplemental data.

## 2.3 Examples of composite gel images

While one may combine multiple images to save on space, one should not combine them without explicitly indicating them. For instance, there are three conditions to meet before combining gel images.

1. The combined gel image should include the molecular weight markers.
2. The combined sites must be made apparent with white or black borders/lines.
3. The composite should be indicated in the Materials and Methods or the legends.

If the above three conditions are met, the combined gel images would be acceptable. Conversely, if these conditions are not met, one may find himself in violation of the submission guidelines instead.



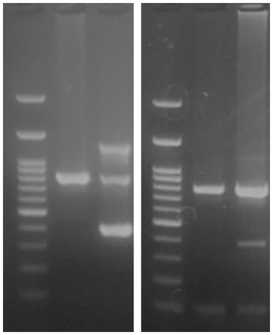


Figure 3-1: An example of an unacceptable composite image Figure 3-2: An example of an acceptable composite image
A white border/line is inserted into the combined site, where each gel image includes the molecular weight markers.

# 3. Inappropriate image processing techniques

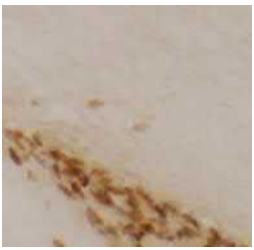
There are many different ways to process biological images for different purposes. Even if the same image to be used, different image processing techniques are to be employed if the research purposes are different. Nevertheless, there are image processing techniques that would impair the quantitativity and the objectivity of the image if they are applied on images as part of experimental data. It is troublesome that many of these techniques are actually used heavily to improve the visibility or appearance of images in certain cases in the fields of engineering or image editing (photo retouching). As a result, even publications or textbooks on image processing now provide explanations on techniques that should never be applied on biological images. Biologists need to carefully consider and select the image processing techniques according to the research purposes. In the next section, the image processing techniques that many biologists should refrain from using are explained.

## 3.1 Copy-paste of images

Copying and pasting of images should not be done carelessly, even if it is not ill-intentioned. Even if it is acceptable to combine gel images together (as mentioned earlier) to save on space or enhance the reader's visibility, scientists are required to clearly indicate and specify it. As mentioned later, the trace of composite images becomes visible by checking the noise, so deliberate image composite is surely detected.

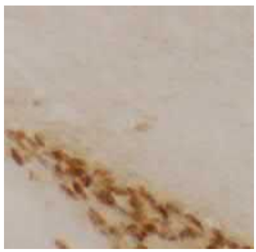
## 3.2 Touch-up function

By using advanced image editing (photo retouching) functions in a software package, it is possible to remove unnecessary debris (such as fine dust appeared in gel images) in experimental data. What constitutes as debris is, however, subjective: it cannot be distinguished whether this is really removing debris, or unfavorable experimental data that does not fit the narrative of the paper. Furthermore, even if the removal of debris improves the appearance of the image, it is not essential at all for scientific data. You should therefore never use touch-up function for biological images.



Original image

A specific area has been touched up



After the specific area has been removed

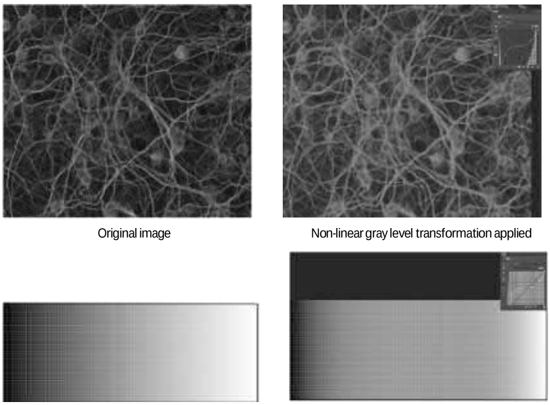
Figure 4-1: An example of auto-repair function

## 3.3 S-shape gamma curve processing (non-linear gray level transformations)

When editing photos with advanced image editing (photo retouching) software, the tone curve is often edited into an S-shape. Adjusting the tone curve will brighten the dark areas, darken the bright areas, and allow you to improve visibility of the entire image. While this function is effective in photo editing, you should be very careful when applying it to biological images.

In digital images, the proportional relationship between the brightness input to the camera and the brightness displayed in the images is important. Take for instance gel images where this proportional relationship is maintained; after background subtraction is applied on background brightness, if the level of brightness is almost double, you may consider the expression level to be nearly doubled as well. However, if the tone curve is adjusted into an S-shape, the originally proportional (linear) relationship will turn into S-shape as well, which would make it impossible to evaluate the brightness quantitatively.

In addition, Nature has set rules that only linear gray level transformations can be applied on gel images, and the Journal of Cell Science (JCS) now requires authors to specify and describe all non-linear gray level transformations, if applied, in the Materials & Methods or legends of their papers.

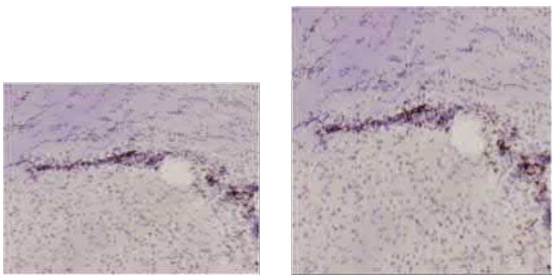


Original image Non-linear gray level transformation applied

Figure 5-1: An example where it is not possible to quantitate the brightness because the tone curve is S-shaped
The brightness spectrum in images gradually change from 0 to 255. This gradation is lost if the tone curve is adjusted.

## 3.4 Distortion

Biological images should never be distorted. As presentation software such as PowerPoint can easily change the aspect ratio of images. you may inadvertently distort the images so as to fit or arrange them in presentation slides or the limited space of papers. Or it could be an unintentional, careless mistake while processing the images. Since students or young researchers who are not particularly aware of this issue may commit such blunders, they should preferably be guided on how to handle images once they are assigned to laboratories.



Original image Distorted image

Figure 6-1: An example where the aspect ratio can be easily changed with PowerPoint etc.
Take note not to distort images so as to fit or arrange them nicely in documents.

### 3.5 Contrast enhancement that would construe as beautification

You may be suspected of removing unfavorable data when beautifying images. If the signal of the important spot is extremely weak and excessive contrast enhancement is unavoidable, you should submit original images as supplementary information.

### 3.6 Processing only one group of comparison images

If there are several images to be compared (*e.g.*, wild vs mutant types, or treated vs untreated groups), the same processing must be applied to all images. Excessive image processing on just one group of images is unacceptable. All image processing techniques and their parameter settings must therefore be standardized.

### 3.7 Processing only a certain part of images

Image processing should be applied across the entire image, and never on a part of the image alone. Even if the colors perceived by the human eye are slightly different from that expressed in the image, one should correct the color across the entire image instead of just changing a specific part.

## 4. Software tools for image fraud detecting

Advanced image editing (photo retouching) software tools now allow you to deliberately and dishonestly remove unfavorable parts from original images. It is difficult to detect dishonest image processing that are intended to mislead readers with a glance. However, such dishonest and unscrupulous image editing will eventually be detected and uncovered. Consistency in the noise level is always present in images; when a certain part of the image is fraudulently edited, the noise there became inconsistent. A simple check with a noise-detecting software will quickly reveal the traces of image editing. Some software tools are available in the market now that help to detect images fraudulently edited.

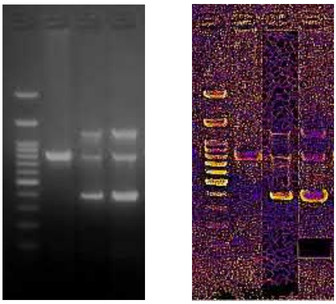


Figure 7: An electrophoretic gel image fraudulently edited (left), and traces of editing revealed on the same image by a software tools that detects fraudulent images (right)
Copy-and-paste traces appear in the third lane, while an insertion trace is found in the fourth lane.


**References**

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2) Hill, E. Announcing the *JCB DataViewer*, a browse-based application for viewing original image files. *The Journal of Cell Biology*, **183**, 969 (2008).
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