Digital images in science

Fair or fraud?

Almost all scientific images are now collected and saved as digital data rather than physical photographs, pen recordings etc., making it far easier to alter their contents. This has led researchers to question what is valid practice and what is inappropriate manipulation of image data[1]. Some have even proposed that there should be rigid rules, but I argue that this is excessively restrictive and will do nothing to curb fraudulent practices.

The first point is that frauds will ignore the rules and cheat, and hence rules of any kind will not prevent the malicious altering of image data. Moreover, cheats lie and will also claim to have followed any rules. Hence rules by themselves do nothing to prevent fraud. Rules do, however, make it more difficult for honest workers to make subtle points and highlight small differences. Consequently, rules penalise the just while providing no effective deterrent to the guilty. At best, rules may prevent researchers from manipulating images inappropriately out of ignorance, but here training is clearly preferable. The difficulty is that training takes time, whereas rules provide the illusion of an instant fix.

The second point is that there is no need for rules about what can and cannot be done to images as long as the processing is described clearly and in detail in the methods section or as a part of a figure legend. Yet with digital micrographs it is common to read that images were collected with a confocal laser scanning microscope or a cooled CCD (couple-charged-device) camera and

that is all the information that you are given; basic information, such as the type of confocal, the size of the confocal pinhole, the numerical aperture of the objective or how the specimen was mounted is all missing. With gels, there is often not even a hint about how the images were produced. This is largely the fault of publishers, journal boards and, through acquiescence at least, the scientific community, many of whom prefer short manuscripts or need low page charges. Moreover, the journals and the scientific community have often compounded this failing by only including tiny illustrations which just show one instance of the specific point of interest, such as images of only a single fluorescently labelled cell or a single row of bands cut from a gel. As a consequence, the system of publishing scientific results has first created a paradise for cheats, then only afterwards started to worry about how to police it, when the answer is that without change you cannot.

Let's (not) cheat

A third problem with rules is formulating them. Take the most simple case possible: a rule which states that you must not make any alterations

to the original image data. Now let's cheat; but let's cheat by following the rule. Figure 1(A) appears to show that actin stress fibres are not formed to any significant degree in Swiss 3T3 cells. However, this is wrong; a processed version of the image (Figure 1B) shows the actin stress fibres very clearly. Moreover, this latter view is the correct one. The image shown was one of a series of optical sections, collected along the axis, with a confocal laser scanning microscope. A comparison of this image with its immediate neighbours made it clear that the fibres were most prevalent at this level. In addition, the serial optical sections were spaced along the axis so that the whole of the specimen was sampled completely, so this really was the clearest view of the stress fibres that could be obtained. The detector gave a linear response to the signal[2] and the image data were essentially free of attenuation and shading over the whole of the field of view[3]. Moreover, there was no saturation or inappropriate biasing of the image data (see below) and the range of the data was spread over almost all (>95%) of the available range of 4096 greyscale values. This means that an almost ideal image that follows the simple rule can provide an incorrect description without any deliberate fraud.

The problem in displaying this image is that the information occupies 12 bits of data (4096 greyscale values), whereas computer screens and printers only display 8 bits (256 greyscale values), at the most.

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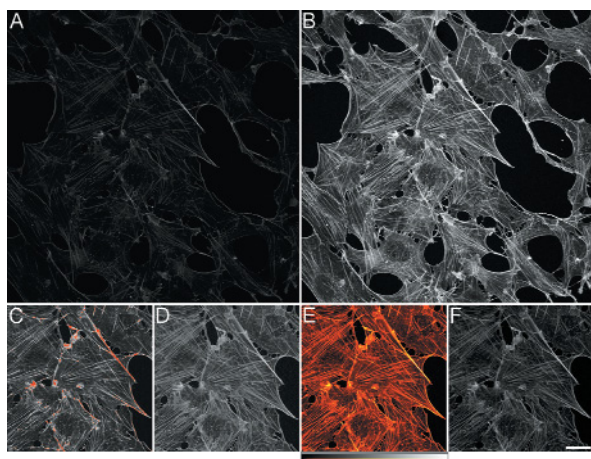(The Ludwig Institute for Cancer Research, London)

Figure 1. The distribution of filamentous actin in Swiss 3T3 cells. The cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, exposed to rhodamine phalloidin to visualize the filamentous actin, and the preparations mounted in Dako mount. The images depict one of a series of lateral views collected along the axis of the microscope at 0.4 μm intervals with a ×40, NA 1.3, oil immersion objective fitted to a well aligned[3] LSM510 laser scanning confocal microscope (Zeiss). Each lateral frame was composed of a matrix of 1024×1024 elements of image data describing the Kalman-filtered average of 16 passes of the laser at a repetition frequency of 0.267 Hz. The specimens were excited by a green helium-neon laser and the emissions filtered through a 560 nm long-pass filter and a pinhole with a diameter of 0.8 Airy units. (**A**) Unprocessed image data. (**B**) Image data subjected to zero-order moderated histogram equalization[8]. (**C**) Subset of the image where the contrast was adjusted using brightness and contrast settings; saturated pixels are highlighted in red. (**D**) Subset of the image where the contrast was adjusted by setting the gamma to 2.5. (**E**) Subset of the image with a contrast-altering look-up table (see panel below figure part) applied. (**F**) The intensity values as shown in (**E**). With the exception of zero-order moderated histogram equalization, the contrast was adjusted using Adobe Photoshop. The scale bar represents 100 μm.

Consequently, only the brightest 256 greyscale values of the 4096 are displayed and, because the intensities in the specimen covered a large range and so much care was taken not to saturate the data, there are very few of those. Moreover, if only 256 greyscale values had been obtained without saturating the data, there would not have been enough information to view the stress fibres clearly. Consequently, in this example the use of 4096 greyscale values was essential if all the views of the specimen were to be interpreted correctly.

To complete my deception by fol-lowing the rules, I propose to get rid of the actin filaments almost completely. If the filamentous actin in the cells is stained with a derivative of fluorescein, the corresponding images are often presented in green. Doing this strengthens the argument that there are no stress fibres present (Figure 2B). When derivatives of rhodamine or Cy3 (cyanine 3) are used, the data are frequently displayed in red, making the filaments almost disappear (Figure 2C). Better, or worse, if derivatives of coumarin (a UV-absorbing, blue-emitting fluorophore) or Cy5 (a red-absorbing, far-red-emitting fluorophore) are employed, images are often shown in blue, which makes it appear that there is scarcely any filamentous actin in the Swiss 3T3 cells (Figure 2D). This conclusion is totally incorrect, but the rules have been followed. Finally, even with an image that depicts the stress fibres very clearly (Figures 1B and 2E), when this is shown in colour there is a very significant loss of detail, especially when the view is displayed in blue (Figures 2F–2H).

Increasing contrast

One way of circumnavigating the problem described above is to stretch the contrast in the image data so that the smallest recorded value is set or mapped to a greyscale value of zero, and the largest value is mapped to a greyscale value of 255, the maximum that can be printed. This process is known as neutral contrast stretching. With the images of the actin filaments (Figures 1 and 2), however, almost all of the range of greyscale values is used already, so this achieves nothing. To display such an image, where there are a few very bright features and a lot of relatively faint ones, demands the use of some form of contrast adjustment that

does not retain proportionality. There are at least four methods. The first is to adjust the brightness and contrast, but to make an acceptable image from the example above means that a significant amount of saturation has to be introduced into the data, effectively discarding all the information about the intensities in the brighter features (Figure 1C, red pixels are saturated). This is clearly far from ideal. The second method is either to expand the contrast in the darker regions of the image while simultaneously compressing it in the brighter regions, or (the opposite) to compress the contrast in the darker regions while expanding it in the brighter regions (Figure 3A). This is usually carried out by altering or adjusting the gamma (see Box 1). As some workers can, and do, argue that this is not an unacceptable practice, the manipulation needs to be considered in more detail.

Adjusting the gamma does not introduce saturation into the data, or bias it inappropriately, giving rise to inverse saturation. Moreover, adjusting the gamma preserves the ranking of the intensities (Figure 3A). All of these are a great improvement over adjusting the brightness and contrast, which can, and usually does, discard information in a highly non-uniform manner. Finally, adjusting the gamma gives a very good representation of the data (Figure 1D). It gives such a good representation that most desktop scanners are configured so that they alter the gamma by default and thus many images of gels are not collected with a neutral gamma. Similarly, the default settings of most of the suites of software that collect images from digital cameras fitted to light microscopes change the gamma by default. Even simply using a CCD camera, including high-quality cooled ones, can

introduce a slight alteration of the gamma of the image data (Figure 3B). This now poses a serious problem about reporting how image data is presented. Researchers who simply go and collect an excellent digital micrograph, using the equipment as supplied and demonstrated by the manufacturer, are not expected to report that the gamma was adjusted by a factor of 1.5, even though the machine did this for them. In contrast, more knowledgeable colleagues who reset the instrument to collect data with a neutral gamma are expected to report that they adjusted the gamma to 1.5 to create the final published images. The images produced by the two workers are identical, yet it appears that the more knowledgeable worker is the less competent (because they needed to use more image processing). This is clearly unfair and highlights a real problem with generating digital images: while researchers are correct to be concerned about what is done to images after they have been collected, they ought to be equally worried about how the data was generated in the first instance.

The third method for altering the contrast in images is to employ a colour look-up table (Figure 1E). With single channels of image data this can be very useful. In the example used here, the intensity component of the pseudo-coloured image gives a good representation of relative intensities in the original image (Figure 1F), but the look-up table was crafted for this particular image to ensure that the quality of representation was high. There are, however, problems with using colour look-up tables. First, while this one was successful, it is very easy to design colour look-up tables which mislead (for an example, see Bolsover et al.[4]). Secondly, it is hard to relate colour to intensity, even

if, as here, a reference bar is supplied. Finally, if the specimen is coloured or different channels of data are to be presented simultaneously using different colours, then colour look-up tables cannot be used.

The fourth method for manipulating the contrast in images is to calculate the adjustments from their intensity histograms, where the most straightforward method is histogram equalization. Histogram equalization starts from the intensity histogram, a graph which describes the number of elements of image data with a particular greyscale value (Figure 3C) for an image (Figure 4A). The computer then attempts to combine some of the greyscale values so that the number of values that depict a particular greyscale value are equalized across the range (Figure 3D), which increases the contrast in the original image (Figure 4B). In this example, histogram equalization was applied sequentially across the two axes of the image data, on a line-by-line basis, to make the binning effects clear in the histogram that resulted (Figure 3D). In practice, the images that result from histogram equalization appear to be processed in a highly exaggerated manner (Figures 4B, 4E and 4H compared with 4A, 4C and 4F) and, essentially, the method does not work well[5–7]. The impact of histogram equalization can, however, be moderated either by blending the equalized view with the original[6,7], or by manipulating the mathematical equations which underlie the process[6,8], yielding a very effective means of adjusting the contrast (Figures 1B, 4D and 4G compared with 1A, 4C and 4F). Moreover, provided that the image data are not saturated or biased inappropriately, the final image produced by means of moderated histogram equalization is practically independent of the
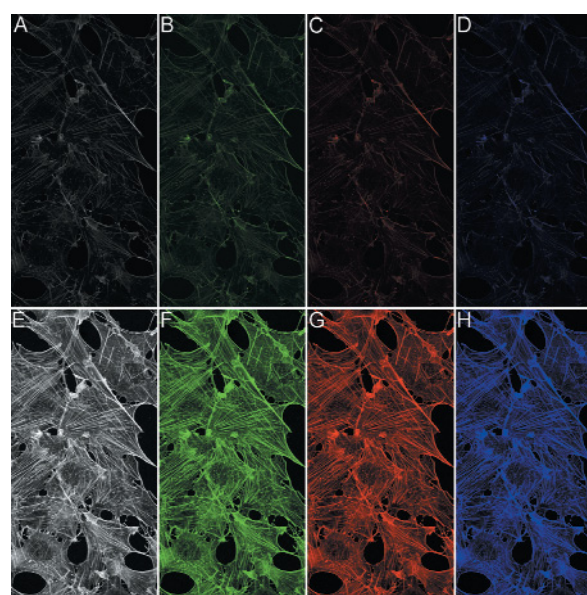

Figure 2. The distribution of filamentous actin in Swiss 3T3 cells shown in different colours to emulate the results of staining with different fluorophores. (A) Subset of the image shown in Figure 1(A). (B), (C) and (D) images have been adjusted to emulate the effects of depicting staining with derivatives of fluorescein (in green), rhodamine (in red) and coumarin or CY5 (in blue). The colours were altered using Adobe Photoshop. (E)–(H) As (A)–(D) but after the application of zero-order moderated histogram equalization. The scale bar represents 100 μm.
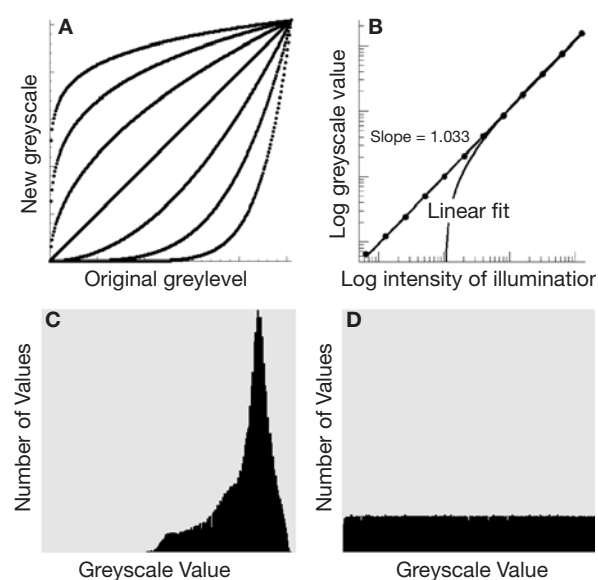

Figure 3. Image gamma and image histograms. (A) The effects of gamma on the contrast in sets of image data. (B) The gamma of a cooled CCD camera; these data were collected for the same length of time and the intensity was regulated by switching the illumination on and off in an otherwise totally dark room. This ensured that the level of dark noise recorded was consistent. (C) The intensity histogram of a digital micrograph depicting histologically stained mammalian cochlea, a part of which is shown in Figure 4(A). (D) As (C), but here histogram equalization was first applied to each row in the set of image data and following this, to each column.
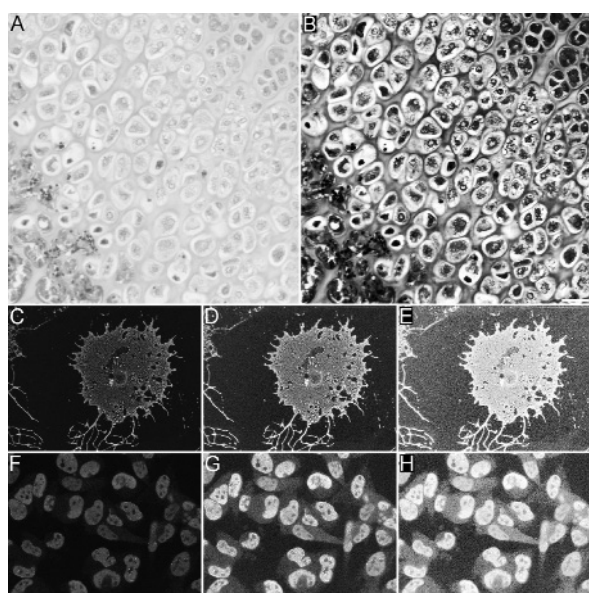
Figure 4. The application of neutral contrast stretching, zero-order moderated histogram equalization and histogram equalization to a series of digital micrographs. (**A**) Part of an image of a section of histologically stained mammalian cochlea from which the histogram in Figure 3(C) was obtained. The image was collected using a Microleaf high-resolution CCD camera, as described previously[9]. (**B**) As (**A**), but following the application of histogram equalization, line by line, across both axes. The scale bar represents 20 μm. (**C**)–(**E**) An oligodendrocyte grown in tissue culture and stained for galactocerebroside immunohistochemically; these data were subjected to neutral contrast stretching, zero-order moderated histogram equalization and histogram equalization respectively. (**F**)–(**H**) Rhabdomyosarcoma cells grown in tissue culture and stained for proliferating cell nuclear antigen immunohistochemically; these data were also subjected to neutral contrast stretching, zero-order moderated histogram equalization and histogram equalization respectively. Images (**C**)–(**H**) were collected using a modified MRC500 confocal laser scanning head mounted over an axioplan microscope[3] fitted with a ×63, NA 1.4, oil immersion objective. The FITC-conjugated second-layer antibodies were excited with the 488 nm line from an argon ion laser and the emissions filtered through a 515 nm long-pass filter and a confocal pinhole with a diameter of 1 Airy unit. Each original lateral frame was composed of a matrix of 768 × 512 elements of image data, describing the sum of 120 passes of the laser at a repetition frequency of 1 Hz. The scale bar represents 25 μm.

adjustments made when the imaging device was set up and used, including gamma adjustments[8]. Finally, it is relatively easy to report how moderated histogram equalization was carried out and there is a fixed, experimenter-independent version, zero-order moderated histogram equalization, which gives excellent results with almost any type of image (Figures 1B, 4D and 4G[8]).

Creating honest images

The first step towards creating a good scientific image is to look at the specimens directly and decide what the results mean (Box 2, steps 1 and 2). The second part of creating good scientific images is to know the instruments that you use and to set them up properly to collect, as far as is possible, unbiased sets of image data that record all the of the information that is available (Box 2, step 3). Busy researchers want simple user-friendly software that makes instruments easy to use instantly, but to make computer programs give apparently excellent images instantly almost always involves compromises. The resulting adjustments make both holiday snaps and scientific data look superb, but often the techniques compromise the integrity of the latter. For example, to hide defects or to make pictures look crisper, many programs apply either Gaussian smoothing or unsharp-masking to all the images collected by default, yet these methods can mask detail and generate false detail, respectively. The third part of creating a good set of image data is to collect a clear description of the results using the minimum number of images (Box 2, step 4; Box 3).

Acquiring high quality digital images is often a slow process and collecting as few images as are absolutely essential is an excellent way of saving time and increasing productivity. Ultimately, I think that you need just two views; the first for your paper in Cell and the second for the subsequent invited review elsewhere.

Up with rules!

If rules about scientific images are needed, they are needed most when the images are collected. I believe that there are, at least, three basic rules here. In general, image data are described using either 0–255 or 0–4095 different greyscale values (even full colour images are composed of three sets of these). All the intermediate greyscale values in these series (1–254 or 1–4094) describe a range of intensities, but zero and the maximum are different because, respectively, they describe less than and greater than a particular intensity without limit, and their presence in a set of image data often indicates a loss of information. Consequently, the first rule is that no element of original scientific image data should ever record the minimum possible value of zero. This avoids negative biasing or inverse saturation. The second rule is that no element of original scientific image data should ever record the maximum possible value, avoiding saturation of the data. With digital micrographs of fluorescently stained specimens, the application of this second rule often means collecting an apparently black screen of data (e.g. Figure 1A), but while the data is being collected it is usually possible to project a contrast-altering look-up table over the image that can be discarded before the data are saved. The third rule is that instruments should always be set so that, as far as is possible, the greyscale values that are recorded are proportional to the intensity of the signal which emanates from the specimen. If information is collected about very bright and very faint features simultaneously, following the three rules above often means that 12 or even 16 bits of data (4096 or 65536 different grey values) have to be collected. This option is offered as a matter of routine with most cooled CCD cameras, confocal microscopes and even domestic desktop scanners,

although this is not always made as clear as it ought to be. When the intensities in images of gels or digital micrographs are to be quantitated, it is essential that the three rules above are followed if the results are to be accurate. If image restoration methods, such as mathematical de-blurring, are to be employed, following the three rules above and collecting at least 12 bits of data is essential. In contrast, when collecting digital micrographs of histologically or histochemically stained specimens, or examining cells under phase contrast, collecting 8 bit greyscale and 24 bit colour data is usually sufficient.

I do not believe that processing images appropriately can be described in a set of rigid rules (Box 2, steps 5 and 6) but there are two rules that help. First, when you are about to change the appearance of some image data you have to ask yourself, 'if I do this could I, even inadvertently, mislead an informed observer?' If the answer to that question is yes, then carrying out that procedure is inappropriate. Secondly, providing that the answer to the first question is no, you have to ask yourself, 'if I do this will I give the observer some extra information?' If the answer to this question is yes, then that procedure ought be carried out and a record made of what was done. You then continue in this manner until you present the observer with as much information as possible, without confusing or misleading them. Finally, because so much information can be lost, colour should never be used when greyscale will do (Figure 2).

Building a safe haven

At the beginning of this article, I stated that I thought that the scientific community had inadvertently constructed an (imaging) paradise for fraud. Rather than relying upon rules, this can be overcome by exploiting digital technology correctly. First, when small sections cropped from larger images are depicted in scientific manuscripts, whether they depict rows of bands in gels or single fluorescently labelled cells, the whole of each of the original images ought be made available in an online digital appendix. The smaller the picture, the harder it is to detect changes that have been made to it. Secondly, methods of improving the contrast that are less sensitive to biases introduced by the instruments need to be developed. A start has been made on this with methods like moderated histogram equalization[6,8]. As mentioned above, this is an effective means of adjusting the contrast in images that is largely independent of how the instrument that was used to acquire the data was configured. Thirdly, more journals could make greater efforts to check that images have not been manipulated. Most routine changes leave small telltale marks in the data and software could be written to detect these semi-automatically. Finally, it would help if the attitude of some scientists and scientific managers towards computers changed; computers and computer software do not turn people into experts. Computers and computer software allow untrained people to do what were, previously, expert jobs; for example, I typed this manuscript myself but this did not turn me into a touch-typist. The spellchecker is a fairly good proof-reader, but it is not prefect. Computers do not make people (not even clever and able researchers) experts and when computer software is designed to attempt this it can all too easily cause scientists to mislead their peers, albeit inadvertently. Using computer-based equipment scientifically takes time, training and, above all, understanding. Perhaps the greatest change needed to improve the quality of scientific images is one of

> **Box 1: The gamma**
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> The brightness of a part of a scene displayed upon a television screen, a cathode-ray-tube-based computer monitor screen, a flat-screen computer monitor and other electronic display devices is increased by increasing the input voltage. Unfortunately, the brightness perceived is not proportional to the input voltage, but is very well approximated by a function of the input voltage raised to a power, where the value of the power varies from screen to screen. In contrast, the level of signal generated by most electronic cameras, including digital cameras, is proportional to the intensity of the signal, or very nearly so (for more details see, for example, http://www.cgsd.com/papers/gamma.html). Consequently, to make the image displayed upon a television screen match the original scene, a correction has to be applied to the input voltage that compensates for the curvi-linear relationship between input voltage and perceived brightness. This compensation is known as gamma adjustment.
>
> A difficulty with adjusting the gamma is reporting what has been done. Most programs describe expanding the contrast in the darker regions with a number that is greater than one and expanding the contrast in the brighter regions with a number that is less than one but, inevitably there are a few programs which reverse this order.

attitude. Either we, the scientific community, have to devote the time needed to learn to use instruments properly, or we need to employ technical staff who are well trained in image recording to do it for us.

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Box 2: Protocol

Check the specimens are prepared correctly.

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Observe the specimens in detail (where possible).

↓

Set the instrument up to collect a neutral image.

↓

Collect the minimum number of images needed.

↓

Examine the images thoroughly to determine what the specimen shows, experiment with the contrast.

↓

Adjust the contrast in a duplicate image so that this clearly illustrates what the specimen means.

Box 3: Viewing a house

Consider a simple example of creating a good image. I want to buy a house with two very similar-sized bedrooms for my two teenage children and a bathroom that is big enough to take a large corner bath. A house is a three-dimensional object, and to display this on the printed page requires that a two-dimensional
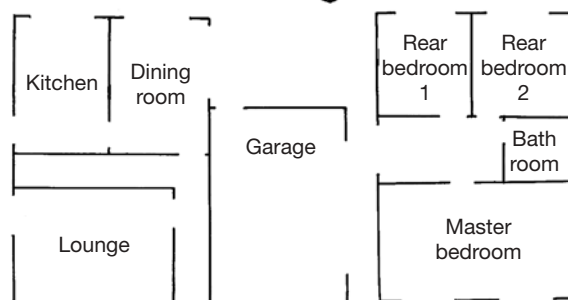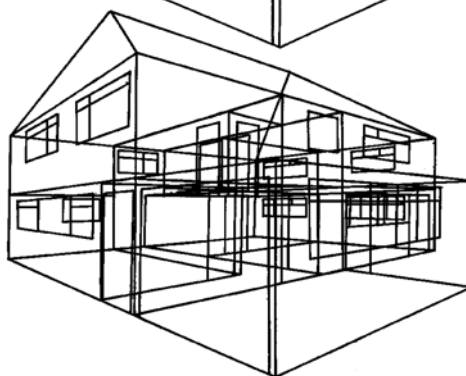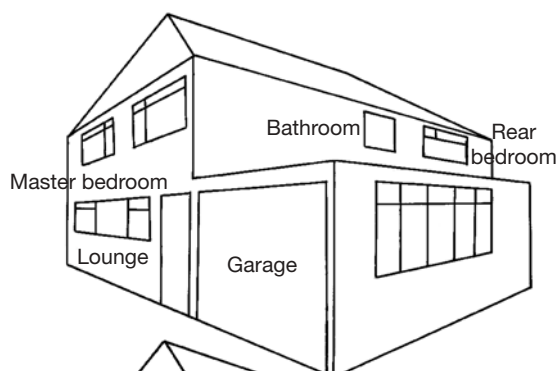
representation of the data is made. First, a surface projection can be generated (upper plate), where it can be seen that the house has two stories, a garage, a sloping roof and lots of rooms, but more data is needed if the size of the rooms is to be studied. To do this with the view shown, it must be made more transparent. The strict rule-based approach demands that you present the observer with all of the data, which can be done by creating a transparent or through-projection: 'acid house' (middle plate). This view is uninformative; it might be improved by rotation but what is needed is a greatly simplified view. In reality, the house only has two forms that differ greatly, the upstairs and the downstairs, and these can be illustrated perfectly adequately using just two simple lateral sections or plans (lower plates), where it can clearly be seen that there are two identical bedrooms for the children but that the bathroom is far too small. In this case, a large 3D-volume set of data, that took an age to collect, was not needed; just two simple sections taken at the appropriate heights and a bit of thought were all that was required.