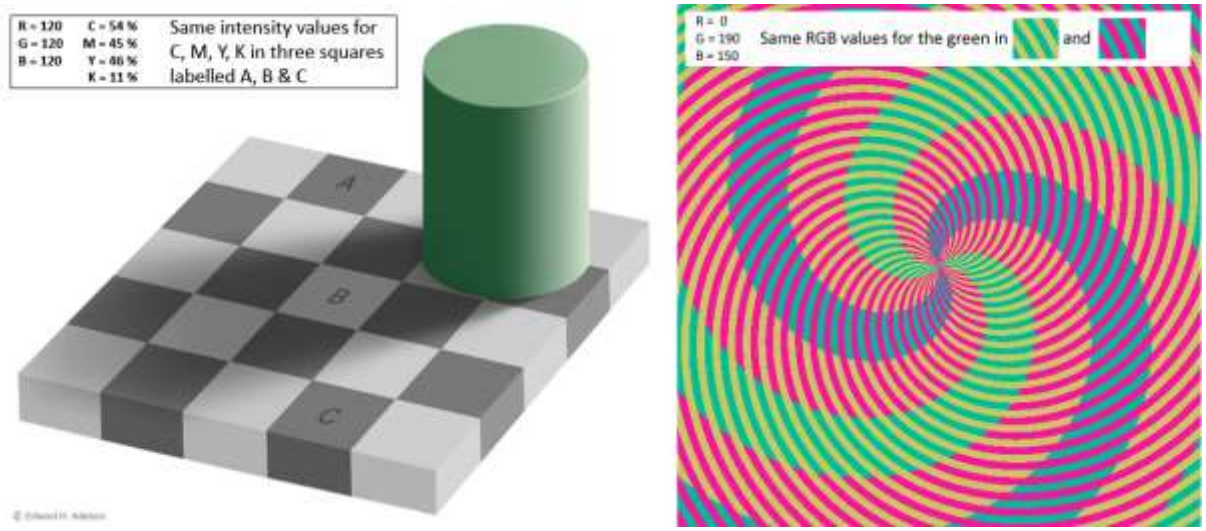
Box 25-1 Merged ‘red-green’ image caveats

There is much published work suggesting that correlation and colocalisation can be inferred from inspecting a merge of red and green pixels, shown as yellow pixels, in the image. This is not possible. Co-occurrence refers to the presence of the emission signal of two (possibly unrelated) fluorophores recorded in the same pixel. With care, conclusions about co-occurrence may be drawn from a merged image of red and green pixels. The following caveats apply:

- First, the pixel *intensities* from both channels determine the merge colour.
- Second, the gamma of the display - which can be set quite arbitrarily – will influence the yellow merge colour.
- Third, whether you use a CCD on a widefield microscope or PMT on a confocal, the camera has a spectral sensitivity curve. Two signals of different wavelength which actually have the same intensity will appear to be of differing brightness.
- Fourth, our eyes are prone to optical illusions, and are very poor at determining colour brightness or intensity. In the figure below, The grey squares of the Adelson illusion (left) are displayed on a computer monitor at the same intensity and hue, whilst the ‘blue’ and ‘green’ colours seen in the spiral are the same colour, so there are three colours, not four. See: https://goo.gl/LM8K5S for an explanation of the Adelson illusion and http://goo.gl/ktWL3p & http://goo.gl/ttAypR from the Fiji and ImageJ analysis webpages for further information on the spiral illusion.
- Fifth, each grey value in the image, which encodes intensity, is independent of the display.
- Sixth, in a two-dimensional image the overlap may suggest correlation, which may, in fact, be co-occurrence because the fluorophores do not overlap axially in the third (unseen) dimension.
- Seventh, if you take this crude approach, it is quite likely that you have not considered Nyquist’s sampling theorem, and have not sampled the specimen properly. In this case, false red-green overlap will lead to a much higher ‘colocalisation’ result than is actually the case.



If we walk into a room entirely painted red, stay for a while and go briefly into another room entirely painted green before returning to the red room, the red colour would seem much brighter on the return visit than before. This is because the red-sensitive cones are depleted during any length of time spent in the red room and are recharged during time spent in the green room when they are not stimulated. On returning to the red room, the hue seems at first much brighter than before, only to appear to diminish in intensity with time.