An Introduction to Microscopy and Digital Imaging

Part 1.4: Digital Images and Imaging Devices

d = \frac{\lambda}{2 \sin \varphi}
In our visual system, a 2-dimensional image of a scene is projected onto the retina, where light stimulates a 2-D array of photoreceptor cells. The response of these cells is integrated in the visual cortex of the brain to generate the perceived image.
Through History, humans have attempted to reproduce what they saw through images
The process became more objective through the invention of photography.
Kodak DCS 100

Released May 1991

1024 x 1280 pixels (1.3 MP)

8 bit grey / 24-bit RGB

200 MB Hard Drive

156 images without compression

$ 20,000 – 25,000

Several pounds

987 units sold
What’s a digital image?

A digital image is a computer file that contains a representation of a visual scene.

This file has been generated with an imaging device, is stored on a hard drive or other medium, and can be displayed or reproduced in a variety of ways, such as a computer monitor or a paper print by using appropriate software.
Natural scenes are composed of an infinite number of points, and a continuous spectrum of intensities, colors, and detail.

$10^0$ m

$10^{-3}$ to $10^3$ m

$10^{-18}$ m

http://micro.magnet.fsu.edu/primer/java/scienceopticsu/powersof10/
To capture and reproduce images, we need to **SAMPLE** them on a medium (photo negative, CCD chip, etc...) with a **FINITE** resolution, brightness, and color range.

**Real world:**
- Continuous and infinite amount of detail
- Continuous and Infinite range of luminosities and colors

**Sampled image:**
- Discontinuous and limited amount of detail
- Limited range of luminosities and colors
To capture and reproduce images, we need to **SAMPLE** them on a medium (photo negative, CCD chip, etc...) with a **FINITE** resolution, intensity, and color range.
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The resolution (detail), luminosity, and color range of the image is determined by the properties of the imaging medium:

- Size of brush and color palette of painter
- Number and size of pixels and bit-depth of CCD camera
It is also limited by the resolution (detail), luminosity, and color range of the display (monitor or printer).

Sampling on recording device

Sampling on display device
• Structural detail (pixels): limited by the number and size of pixels you can pack on a chip (engineering and commercial problem).

• Color/luminosity (levels of grey/number of colors): limited by characteristics and type of the CCD chip, electronics, file size, characteristics of the display (monitor) and printer (engineering and commercial problem).
In a digital image, a visual scene is captured and reproduced as a two dimensional bit map, i.e. a 2-D array of points.
Two dimensional bit map

H x V pixels (e.g. 1200 x 1000)
A numerical value is assigned to each pixel. This numerical value represents the brightness, and/or color of the image at that location.
A binary (1-bit) image is composed of only two values ($2^1$), represented in the image file by zeros (0) and ones (1). The “0” and “1” values are often displayed as black and white. Such images do not look very realistic to us...
8-bit images contain $256 \left(2^8\right)$ intensity values (0-255). These values are typically represented as shades of grey.
Number of shades of grey

2

8

64

256
8-bit images appear relatively natural to us... 256 intensity levels are sufficient to be perceived as continuous by our eyes (i.e. have enough contrast or dynamic range)
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8-bit is the most widely used standard for monochrome images

Pixel values are represented by a number from 0 to 255
A **look-up table** (LUT) is a table that assigns a specific grey (or color) value to a specific numerical value, and defines how each pixel in a digital image is to be displayed.
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8-bit images do not need to be displayed as grey

Example of an 8-bit color LUT (256 colors)
An eight bit image can contain only 256 intensity values or colors.

Early monitors could display 256 shades of grey, and later 256 colors

This is typically not enough to represent a typical natural scene

It’s also quite limiting for scientific applications....
A 16-bit file can contain up to 65,536 intensity values ($2^{16}$)

Scientific cameras are typically 12-bit, 14-bit or 16-bit
Color images are generally encoded and displayed as an overlay of Red, Green, and Blue channels, with 256 levels (8-bit) per channel.
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This is the widespread 24-bit RGB format

(= 3 channels x 8 bits)
In a 24-bit RGB image, each pixel is represented by three 8-bit values. There are therefore $256 \times 256 \times 256 = 16,777,216$ possible colors. This is often advertised as “millions of colors”) for PC monitors.
Mixing equal amounts or Red, Green and Blue light creates white.

Mixing equal amounts of two of the primary colors creates a complementary color.

e.g. yellow is created from equal parts of red and green.
By mixing variable amounts of red, green and blue light, one can create millions of different colors.
In a PC monitor, this is achieved by using little Red, Green and Blue dots lit to different levels. One set of three R,G,B dots corresponds to one pixel.
<table>
<thead>
<tr>
<th>Bit depth</th>
<th>Levels</th>
<th>Color/Texture</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-bit</td>
<td>256</td>
<td>(monochrome)</td>
</tr>
<tr>
<td>10 bit</td>
<td>1024</td>
<td>(monochrome)</td>
</tr>
<tr>
<td>12 bit</td>
<td>4096</td>
<td>(monochrome)</td>
</tr>
<tr>
<td>16 bit</td>
<td>65536</td>
<td>(monochrome)</td>
</tr>
<tr>
<td>24 bit RGB</td>
<td>$256^3$</td>
<td>(color)</td>
</tr>
<tr>
<td>36 bit RGB</td>
<td>$4096^3$</td>
<td>(color)</td>
</tr>
</tbody>
</table>

File size: (number of pixels) x (number of bytes) [1 byte=8 bits]

E.g. 512x512 image in 24-bit RGB:

File size = 512x512 x 3 = 786,432 bytes = 786 kB
Digital file:

Header: contains information about the image, the file, and the software used, such as “24-bit RGB TIFF image, uncompressed, created with Photoshop, on 12/25/2012. Image contains 600 horizontal by 1000 vertical pixels).

Image file information: list of pixel intensity/color values
File formats: 
- TIFF: tagged image file format, generally uncompressed
- RAW/DNG: (original information from camera)
- JPEG
- GIF/PNG
- Proprietary file formats (Zeiss, DeltaVision, Photoshop)

TIFF: most “quantitative” file format

Proprietary imaging file formats: RAW or variants of the TIFF format

Always save files in proprietary or TIFF format

Avoid compressed file formats (JPEG, GIF, etc...):

Repeated cycles of encoding/decoding will alter data
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Part 1.4 B: Imaging Devices

\[ d = \frac{\lambda}{2 \sin \theta} \]
In microscopy and other scientific applications, there are two main types of image acquisition methods:

2-D Imaging device

CCD chip or cMOS chip

Scanning device

Photomultiplier, CCD, etc...
The CCD chip or cMOS chip

This is the central component of most digital cameras

A CCD chip is a 2-D array of small photodetectors. Each photodetector corresponds to 1 pixel.

The CCD chip is an imaging device... it records a 2-D map of an image projected onto its surface.

In this respect, a CCD chip is very similar to the retina of the human eye (or to photographic film).
How a pixel on a CCD chip works

Pixel at rest

Exposure to light creates a charge (separates electrons)

Electrons are recycled and current is measured

Pixel at rest ready for next exposure

Layers of silicon
In a CCD camera, each pixel is like a bucket that collects electrons when exposed to light... the more light, the more electrons are accumulated...

The maximum number of electrons you can accumulate is called the full well size. It depends on the size of the pixels. Typically, the bigger, the better....
After each exposure, the charge accumulated in each pixel is measured and converted to a number (digitized).

For example, for a CCD with a full well size of 40,000 electrons and 8-bit conversion, 156 electrons would correspond approximately to one intensity unit in the final image (40,000 / 256)
Some factors to consider in a scientific grade CCD camera

**Quantum efficiency**: fraction of photons converted to electrons

- 60-90% in modern cameras

**Dark current**: baseline signal of the camera in the dark

- can be reduced by cooling the camera

**Read noise**: basically the uncertainty or error bar of the camera’s output
Typical specs of a scientific grade CCD camera: Photometrics Coolsnap HQ²

<table>
<thead>
<tr>
<th>Feature</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image type</td>
<td>Monochrome</td>
</tr>
<tr>
<td>Output</td>
<td>12-bit/14-bit</td>
</tr>
<tr>
<td>Resolution (chip size)</td>
<td>1392 x 1040 (1.4 Megapixel)</td>
</tr>
<tr>
<td>Pixel size</td>
<td>6.45 x 6.45 microns</td>
</tr>
<tr>
<td>Full well</td>
<td>16,000 e⁻</td>
</tr>
<tr>
<td>Quantum Efficiency</td>
<td>~ 60%</td>
</tr>
<tr>
<td>Cooling</td>
<td>-30°C</td>
</tr>
<tr>
<td>Read Noise</td>
<td>4.5 e⁻ rms @ 10 MHz</td>
</tr>
<tr>
<td>Dark Current</td>
<td>0.001 e⁻ / pixel / second at -30°C</td>
</tr>
<tr>
<td>Interline Sony ICX285 chip</td>
<td></td>
</tr>
<tr>
<td>About 9 mm x 7 mm</td>
<td></td>
</tr>
<tr>
<td>Readout</td>
<td>90 ms (11 fps)</td>
</tr>
</tbody>
</table>
Typical specs of a scientific grade CCD camera: Photometrics Coolsnap HQ²
Typical specs of a scientific grade CCD camera: Photometrics Coolsnap HQ²

Full well: 16,000 e-
Output: 12-bit/14-bit

> ~ 4 e- per intensity value at 12-bit
> ~ 1 e- per intensity value at 14-bit

Read Noise: 4.5 e- rms @ 10 MHz

Dynamic Range: 16,000 / 4.5 = 3,555

Dynamic Range = Full well size / Read Noise

Read noise = error bar

Two values can be distinguished if their difference is greater than the error bar
Dark Image acquired with Coolsnap HQ (exposure in the dark)

- Count: 1447680
- Mean: 122.559
- StdDev: 1.014
- Bins: 256
- Min: 117
- Max: 128
- Mode: 123 (509084)
- Bin Width: 0.043
To measure read noise of a CCD camera:

1. Collect and average several frames.
2. Subtract one frame.
3. The residual noise is the read noise.

![Read noise graph](image)
A good CCD camera will have a very tight histogram when a dark image is collected.

The read noise will be small (a few counts), and the standard deviation will be small (very uniform image).

A bad camera will have high read noise, large standard deviation, and occasionally random “dark” or “hot” pixels, i.e., pixels that do not respond, or pixels that give an unusually high signal.
CCD cameras are monochrome; they only record illumination intensities (number of photons). They do not “see” color.

How do you record color?

Color is recorded by acquiring three separate images for red, green, and blue wavelengths
Color is recorded by acquiring three separate (grey) images for red, green, and blue wavelengths, and overlaying them as red, green, and blue layers.
The most common type of color CCD cameras use a Red Green Blue Bayer Mask. Each color is recorded by only a subset of pixels. The missing values are interpolated. Inherently less sensitive and less resolution, i.e. not ideal for fluorescence...
The Bayer mask system is somewhat analogous to the human retina.
Color images can also be recorded by splitting the light onto Red, Green, and Blue components, and recording each on a separate chip (3-chip CCD)

These are better cameras, because information for each primary color is recorded at all pixel locations... however, they are more complex and more expensive
Finally, color images can also be recorded by taking three sequential images through a Red, Green, and Blue filter, onto a single monochrome chip.

These are cheaper than a 3-chip CCD, and provide excellent color reproduction and resolution. They’re a good option for doing both fluorescence and transmitted light microscopy. However, they are not good for moving objects....

Tip: to collect a true color image on a monochrome fluorescence camera, take three exposures through the blue, green and red fluorescence filters. Merge images and adjust color balance.
To learn more about CCD cameras:

http://www.photometrics.com/resources/learningzone/
In microscopy and other scientific applications, there are two main types of image acquisition methods:

2-D Imaging device

- CCD chip or cMOS chip

Scanning device

- Photomultiplier, CCD, etc...
In microscopy and other scientific applications, there are two main types of image acquisition methods:

**Scanning device**

With a scanning imaging device, the pixels that make up one image are not acquired all at once as with a CCD camera, but rather are acquired sequentially through a scanning mechanism.
In old-fashioned televisions, before the era of digital displays, the image was formed by scanning a modulated electron beam onto a phosphor screen.

The intensity of the electron beam was modulated as it was scanning the screen, and therefore the phosphors in the screen were brighter or darker at different locations, depending on the intensity of the beam.
A flatbed scanner is a typical scanning device
Generalized scanning device

From GE’s Fluorescence Handbook
Scanning mechanism of the Typhoon Trio

The Typhoon uses a hybrid mechanism, with both mechanical and optical scanning.

From GE’s Fluorescence Handbook
Scanning mechanism of a Confocal Microscope (Point Scanning Confocal)

Source: Carl Zeiss Micro-Imaging
Major difference between Camera imaging and scanner imaging:

With an imaging device (such as CCD chip, photographic film, etc...), an image is projected onto a 2-D detector (chip) and all the pixels are recorded at once on the imaging substrate and transferred to the computer. There is a simple relationship between the original scene and the recorded image.

With a scanning device, the information for each pixel is read sequentially, and the image is created from all the readings by the imaging software. Sampling of the image is done over space, but also over time, i.e. intensity readings are taken at specific time intervals, as the device is scanning the sample.
With a scanning device, such as a confocal, it is critical that all the imaging parameters (scan speed, image size, ...) are set correctly. Otherwise, structures will be missed, and/or there may be image artifacts.