THE UNIVERSITY OF QUEENSLAND AUSTRALIA | CREATE CHANGE

Introduction to Image Processing

Nicholas Condon (n.condon@uq.edu.au)

Light Microscopy Officer

ACRF: Cancer Biology Imaging Facility
Institute for Molecular Bioscience

1

Institute for Molecular Bioscience

THE UNIVERSITY OF QUEENSLAND AUSTRALIA

Why perform image Analysis?

'More than just a pretty picture…'

Microscopy based experiments can provide you with both **Qualitative** (pretty pictures) as well as **quantitative** data (meaningful numbers and statistics).

Many journals are now asking for quantification of image data, and knowing what you can and can't do, as well as how to do it is vitally important!

Introduction to Image Analysis   **CRICOS code 00025B**   2

2

What is an image?

An image is an array of pixels with varying intensity levels

110	95	76	47
128	114	89	62
147	123	94	79
172	141	109	88

Each pixel represents an intensity value.
Lower value pixels are darker, while lighter pixels have higher values

Pixels = 2D
Voxels = 3D

3

Image Types

In microscopy there are two **types** of images:

-Grayscale images (fluorescence)

-RGB colour images (brightfield)

The range of the pixel intensities depends on the **Bit Depth** of the images

8-bit images have a dynamic range of 2^8 (0-255) -Visualising

12-bit images have a dynamic range of 2^{12} (0-4095) -Quantifying

16-bit images have a dynamic range of 2^{16} (0-65535) -Quantifying

For example Intensity values for **8-bit greyscale images** range from 0 to 255

(black>grey>white) with varying levels of grey for each pixel intensity.

4

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Microscope Images have dimensions

An Image can have multiple dimensions (width, height, depth, channel, time)

We use standard terms to describe image datasets;

Width = x

Height = y

Depth = z

channel = c

Time = t

For example; a 2D image stack (xy) = (512 x 512)

3D image stack (xyz) = (512 x 512 x 10)

3D image stack with multiple colours (xyzc) = (512 x 512 x 10 x 3)

3D image stack with 3 colours & 20 timepoints (xyzct) = (512 x 512 x 10 x 3 x 20)

*note that the order of your dimensions isn't the same for every acquisition and having the file read incorrectly can make image analysis difficult.

Different microscopes order their files differently; metadata within the image file (or accompanying .xml file) describes the order. **Converting files may loose this information

Introduction to Image Analysis

CRICOS code 00025B

5

5

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

What about multi-coloured images (RGB)?

An RGB image is 24-bit, with an array of three 8-bit colour ranges representing the intensity levels for the Red, Green & Blue images.

Red

12	15	30	29
15	20	41	36
19	27	48	40
29	34	41	25

Green

110	125	132	117
106	124	130	115
107	126	129	112
112	115	125	106

Blue

109	98	46	33
107	101	50	34
107	106	51	36
104	74	48	27
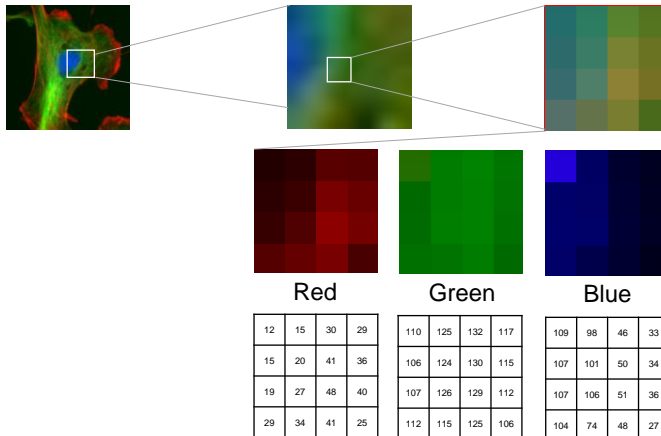
Each 'colour channel' is actually a greyscale image, pseudo-coloured via a look up table (LUT).
RGB images can be flattened (resulting in 1 channel) made up of 3 values (e.g. 12, 110,109)

Introduction to Image Analysis

CRICOS code 00025B

6

6

3

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

What makes a good image?   Image Size

Microscope hardware (objectives & optics) will determine the resolution possible with the system, however the size of the output image is often adjustable.
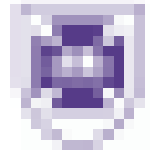
Image Size refers to the **height** and **width** of the image in pixels.

For example:
- Camera chip size (eg 4MP = 2048 x 2048)
- Scan area on LSM confocal (user + system adjustable, 1x1 up to 8096 x 8096)

Ensuring you have enough pixels in your image is important for resolving structures.

(but too many may be a waste of time)

15 x 15

31 x 31

151 x 151

Introduction to Image Analysis

CRICOS code 00025B

7

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

What makes a good image?   Signal:Noise & Dynamic Range

SNR is a measure of the level of signal to the level of background noise
- Higher ratio is better

Also important to maximise the range of data – i.e. spread the data out over the whole dynamic range of the bit-depth you are using
- Will help with image analysis later

- A is easier to see the signal and will be far easier to perform threshold analysis on later

Introduction to Image Analysis

CRICOS code 00025B

8

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA
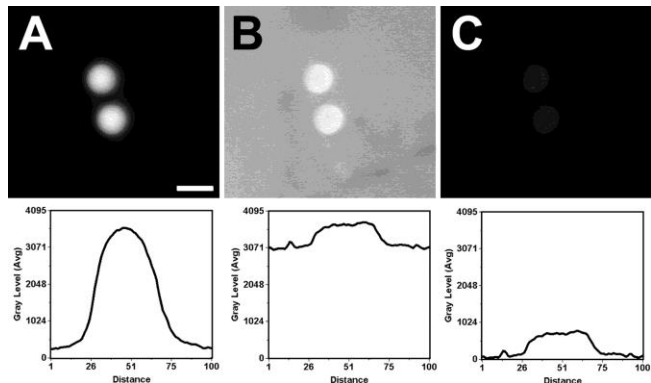
What makes a good image? Image Saturation

Important not to over-expose your image
- **Data will be lost by saturation**
- Lose the ability to differentiate intensity levels (structures)
- Plan for the future as well – not just what you are interested in now!
    - *For example, a time course of a peptide entering the cell/tissue is likely to increase in intensity over time! Set up your imaging parameters using a later timepoint.*

Introduction to Image Analysis   **CRICOS code 00025B**   9

9

THE UNIVERSITY OF QUEENSLAND
AUSTRALIA
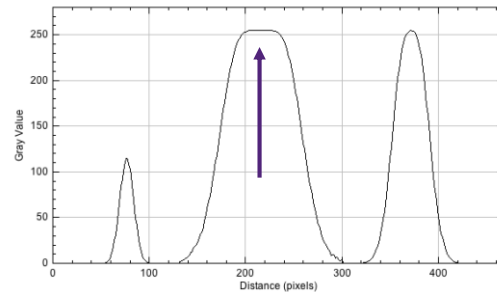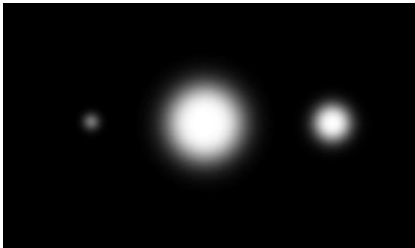
What makes a good image? Consistency

Vital to be consistent with image acquisition settings

Exposure time (camera based) or Gain (PMT based) needs to remain the same across all images

Same applies to other settings like lamp intensity, laser power, pinhole size etc

Sample A   Sample B   Sample B – ↑ exposure

Introduction to Image Analysis   **CRICOS code 00025B**   10

10

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA
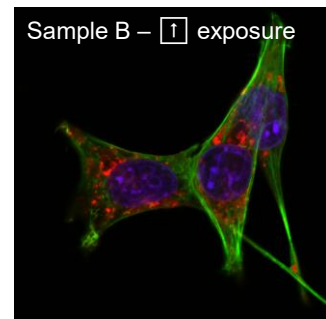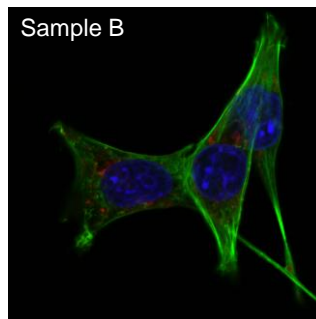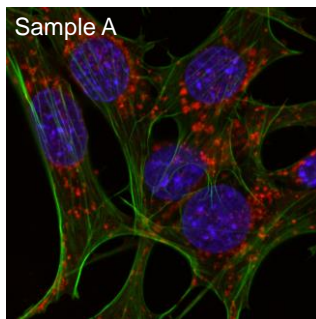
How do I capture a good image?   Sample Prep

Crap in = Crap out

- Use the right coverslip/dish/glass for your experiments. **Number 1.5 (#1.5)** coverslips are what objectives are designed for (0.16 – 0.19mm thick).

    Note what is written on the objective (Hint all IMB Microscopy's objectives are 0.17)

- Use modern dyes and fluorophores (Alexa-, ATTO-, STAR-, mEmerald,mNeonGreen,) for brighter and increased photo-stability.
- Clean your slides and coverslips with 70% ethanol prior.
- Seal and mount your slides with clear nail polish and use a reputable mounting media
- Image fixed slides promptly (samples fade over time!)
- Use the current media for live imaging (buffered, phenol red free)

Introduction to Image Analysis   CRICOS code 00025B   11

11

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

How do I capture a good image?   Optical Resolution

The optical resolution of a microscope is determined by the objective & wavelength.

The Reyleigh formula for resolution   =   0.61 x (λ / NA)

where λ = wavelength in μm

NA = Numerical aperture of the objective used.

For example:

Resolution   = 0.61 x (0.515 / 1.4)

= 0.224 μm (Using a 1.4NA objective with Green$_{[515]}$ emission)

= 224 nm (therefor pixels should be about 100 nm)

The higher the NA of the objective (and lower wavelength) the more resolution is achievable.

Note: Know the size of the object you wish to image and therefore what resolution you need and use that to determine the NA of the objective needed and therefore which microscope you will need to book.

Introduction to Image Analysis   CRICOS code 00025B   12

12

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

How do I capture a good image? Nyquist Sampling

Microscopy images are digital representations of analogue (natural) events. We must therefore sample them at a rate that truly represents what is observed.

Nyquist sampling rate is defined as being twice that of the spatial frequency detectable.

> *In a perfect setting this would be twice that fo the theoretical limit of the optics (Abbes Limit) or 0.11 μm sized pixels, although is more often larger due to lower NA objectives. (**Optical systems and resolution could be a whole workshop).*

0.83x 1.3x
Undersampled

2.3x
Just enough

10x
oversampled
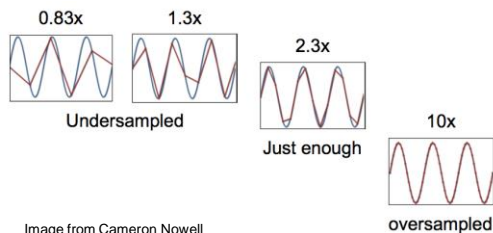
Image from Cameron Nowell

This applies for XYZT axis of imaging!

Introduction to Image Analysis CRICOS code 00025B 13

13

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

How do I capture a good image? Image Size

Image resolution is the shortest distance between two objectives that can be separated by the detection systems.

Pixel resolution describes the image size

> *For example* an image 2048 pixels wide x 2048 high has 4,194,304 pixels.
> This can be described as being 4.19 megapixels

Spatial resolution refers to the imaging systems ability to differentiate two objects

> *For example the number of pixels and the distance between them is known.*
> *This is related to the optical resolution of the microscope used, and has a theoretical limit of 0.22 μm (Abbe Limit).*

Spatial frequency describes the rate changes in brightness (pixel intensity) change throughout the image.

Introduction to Image Analysis CRICOS code 00025B 14

14

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

How do I capture a good image? Intensity/Histogram

Pixel intensities are often represented in microscope software and image image analysis programs as histograms.

These are frequency distributions of the individual pixel intensity for every single pixel of your image.

Dark Pixels ◄ ▲ ▲ ▲ ► Light Pixels

Range Indicators

Can tell you if your image is under or over exposed (saturated).

Zeiss

Leica

Introduction to Image Analysis

CRICOS code 00025B

15

15

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

How do I capture a good image? File Type / Format

Many different file formats exist for image data; know which ones to use!

JPEG is a **LOSSY** format and should be avoided at all costs
Compression in the JPEG file format means information is lost (mainly resolution).

Tiff (or OME-TIFF) is **Loss-less** and is a good common file-format. (but may loose meta-data)

HDF-5 is a new format that is also **Loss-less** when it compresses data.

Microscopy vendors often have proprietary image formats. All major commercial systems produce files that are 'Bio-Formats' compatible.
> *.czi, .oir, .sld, .nd2, .lsm are all openable by ImageJ/FIJI as long as Bio-Formats is up to date.*
> *Often the vendor has free software to open and view data-sets.*

**Some visualization formats (.ims, or .sis) are protected and can only be opened in commercial software (note FIJI)

Introduction to Image Analysis

CRICOS code 00025B

16

16

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Microscope Images have dimensions – Modern Microscopes

As microscopes get more complicated so do their dimensions

New technologies and imaging modalities have meant that a single captured image file can have <u>unlimited</u> dimensions!

For Example:
-Zeiss Apotome images will have 5x phase shift images /slice/channel
-Zeiss Airyscan images will have 32x 'channels' /slice/channel
-Spectral confocal (Lambda) scans can have an image /wavelength/slice/channel
-Leica SP8 with WLL can perform a 'Lambda, Lambda' scan =~ 40,000 images/slice
-Multi-position and tiling images can all be stored within the same image file
-SIM and SRRF store frequency space, and fluctuation data within the raw data

<u>Anything can be a dimension!</u>

Programs such as FIJI are capable of an unlimited number of image dimensions as well.

CRICOS code 00025B 17

17

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Breaking the Optical Resolution Limit

Despite the Rayleigh limit to resolution being dependent on the N.A. of objective & wavelength (~240nm with modern objectives), many methods exist to break this limit - called SUPER-RESOLUTION microscopy.

STED – uses a depletion laser doughnut to limit the excitation area to a minimum (30nm)

Airyscan – uses a special 32-detector lens to image different parts of the light pattern (120nm)

TIRF – limits excitation in Z to around 100nm

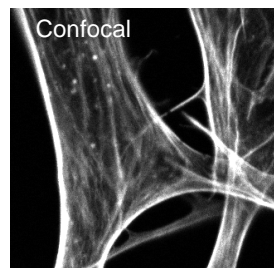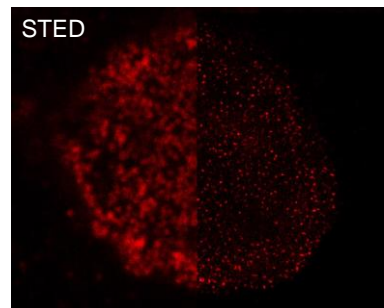*PALM/STORM – Single Molecule resolution methods using statistical analysis of "blinking" signal*

STED

Confocal

Airy-scan

CRICOS code 00025B 18

18
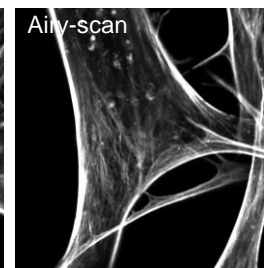
Image Analysis Best Practices

19

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

NHMRC – Code of Conduct

A strong research culture will demonstrate honesty and integrity

Researchers must foster and maintain a research environment of intellectual honesty and integrity, and scholarly and scientific rigor.

Researchers must respect the truth and the rights of those affected by their research

Research publications should be complete and, where applicable, report negative findings and results contrary to the hypothesis

Researchers must take all steps to ensure their findings are accurate and properly reported

https://www.nhmrc.gov.au/about-us/publications/australian-code-responsible-conduct-research-2018

Introduction to Image Analysis

CRICOS code 00025B

20

20

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Basic Rules for handling and editing microscopy images

1. Scientific Digital Images are Data that can be Compromised by Inappropriate Manipulations

2. Manipulation of Digital Images Should only be Performed on a Copy of the Unprocessed Image Data File (Always Keep the Original Data File Safe and Unchanged!)

3. Simple Adjustments to the Entire Image are Usually Acceptable

4. Cropping an Image is Usually Acceptable

5. Digital Images that will be Compared to one Another Should be Acquired under Identical Conditions, and any Post-acquisition Image Processing Should also be Identical

6. Manipulations that are Specific to one Area of an Image and are not Performed on Other Areas are Questionable

Further reading is available from **Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images** Douglas W Cromey 2010, Sci Eng Ethics doi: 10.1007/s11948-010-9201-y

Introduction to Image Analysis

CRICOS code 00025B

21

21

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Basic Rules for handling and editing microscopy images

7. Use of Software Filters to Improve Image Quality is Usually not Recommended for Biological Images

8. Cloning or Copying Objects into a Digital Image, from Other Parts of the Same Image or from a Different Image, is very Questionable

9. Intensity Measurements Should be Performed on Uniformly Processed Image Data, and the Data Should be Calibrated to a Known Standard

10. Avoid the use of Lossy Compression

11. Magnification and Resolution are Important

12. Be Careful when Changing the Size (in Pixels) of a Digital Image
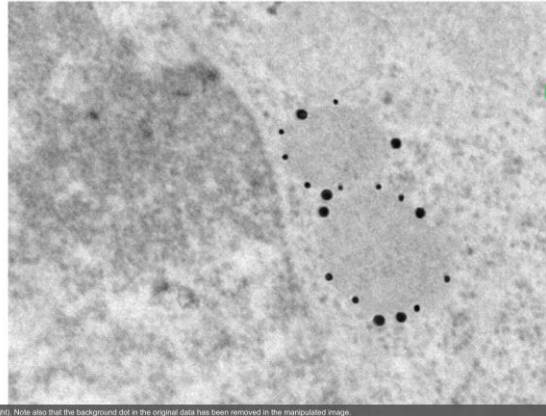
Further reading is available from **Avoiding Twisted Pixels: Ethical Guidelines for the Appropriate Use and Manipulation of Scientific Digital Images** Douglas W Cromey 2010, Sci Eng Ethics doi: 10.1007/s11948-010-9201-y

Introduction to Image Analysis

CRICOS code 00025B

22

22

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Example of image manipulation – selective enhancement
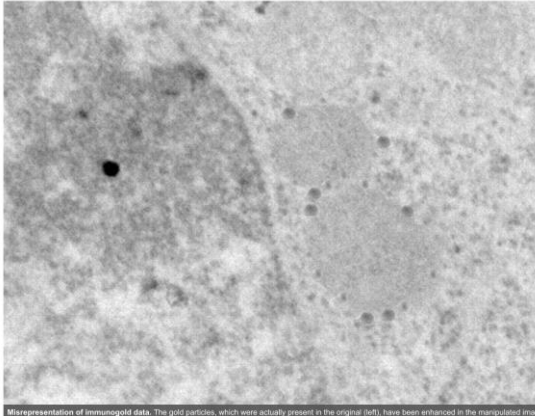
Original image

Manipulated image

Image from Mike Rossner, and Kenneth M. Yamada J Cell Biol 2004;166:11-15

Introduction to Image Analysis
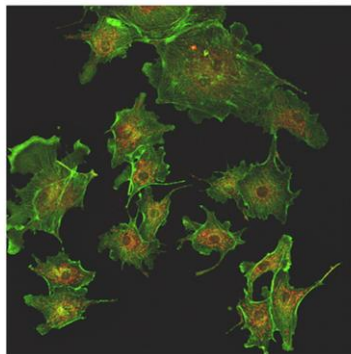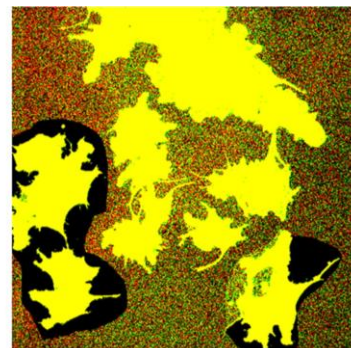
CRICOS code 00025B

23

23

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Example of image Manipulation - Cropping

Manipulated image

Manipulation revealed by contrast adjustment

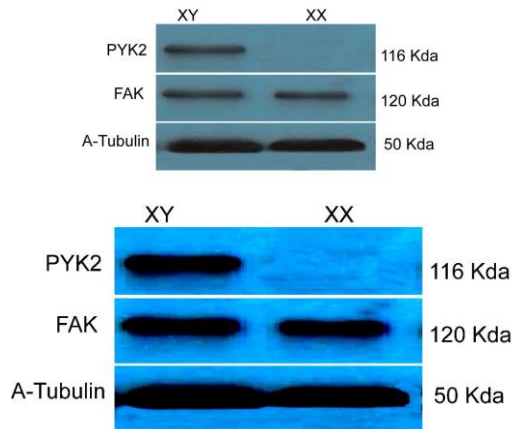Image from Mike Rossner, and Kenneth M. Yamada J Cell Biol 2004;166:11-15

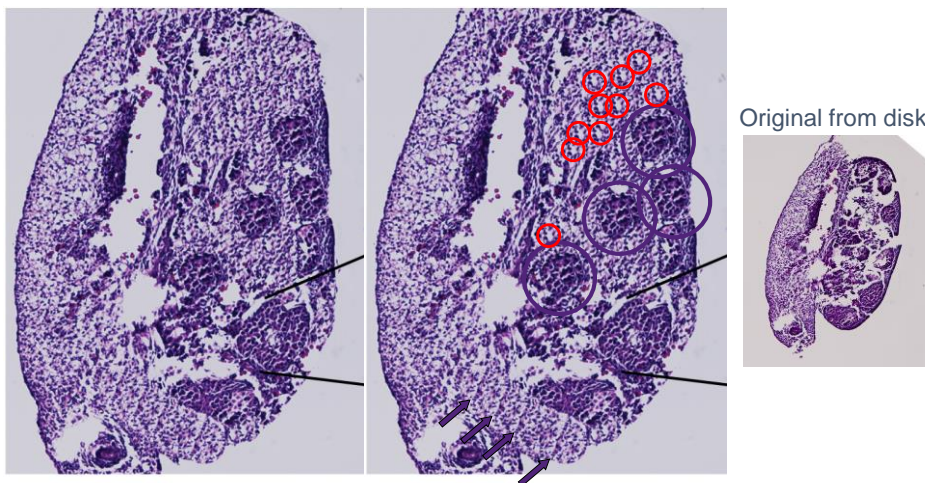Introduction to Image Analysis

CRICOS code 00025B

24

24

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA

Forensic Image Analysis Extraordinaire

https://twitter.com/MicrobiomDigest/status/1234541598404272129?s=20

Introduction to Image Analysis · CRICOS code 00025B · 27

27

THE UNIVERSITY
OF QUEENSLAND
AUSTRALIA
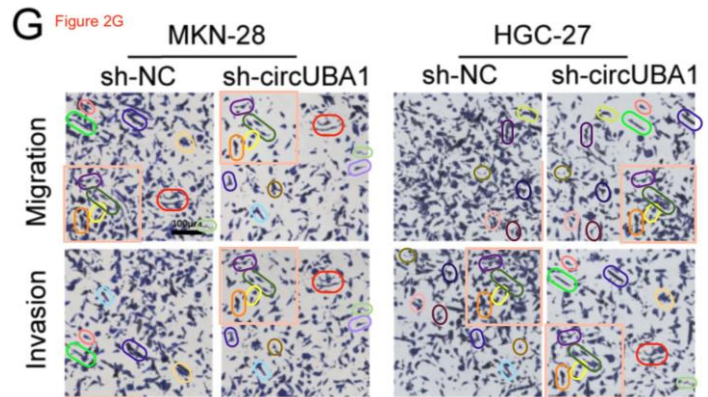
Saving and backing up your data

All images are required to be kept and backed up as per NHMRC guidelines.

- Image files should not be left on the microscope or your laptop **(not backed up)**
- Instead copies of your original data should be stored on a backed-up server/cloud (eg UQ-RDM) **(backed up)**
- Images should be left in their original file formats:
  - -Contains all the meta-data about the image (bit-depth, scale, etc)
  - -ImageJ/FIJI using Bio-formats can open any file-type from our microscopes

Develop a naming convention
- Consistent
- Needs to be understandable by other researchers
  - *e.g. Fluorophore1-Protein_Fluorophore2-Protein_#sequence*
- Folders should be named 20191205 (for the 5th December 2019) to ensure correct sorting order.

Introduction to Image Analysis · CRICOS code 00025B · 28

28

Thank you

Dr Nicholas Condon | Light Microscopy Officer
ACRF: Cancer Biology Imaging Facility
Institute for Molecular Biosciences
n.condon@uq.edu.au
07 3346 2042 | 0400 909 510

facebook.com/uniofqld

Instagram.com/uniofqld

@DrNickCondon

29