

Live Cell Imaging





- Outline -

Hardware –

Inverted Microscope : LeicaDMI4000

Incubation Chamber

Acquisition Device : Roper HQ2

Software –

MetaMorph





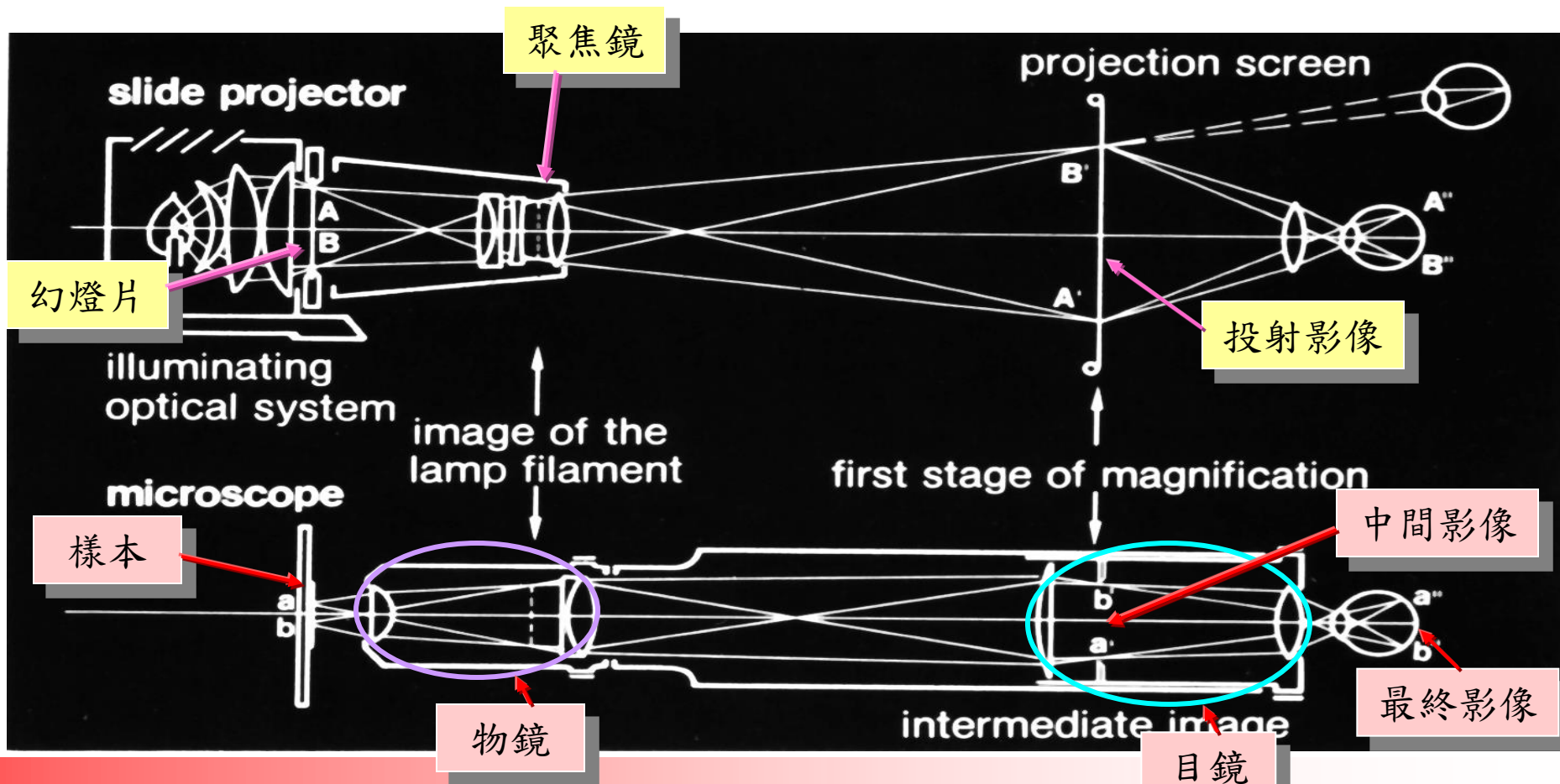
Light Microscopy

- **Image formation**
- **Optics**
 - **Aberration**
 - **Objectives**
- **Bright Field, Phase Contrast**
- **Fluorescence Microscopy**



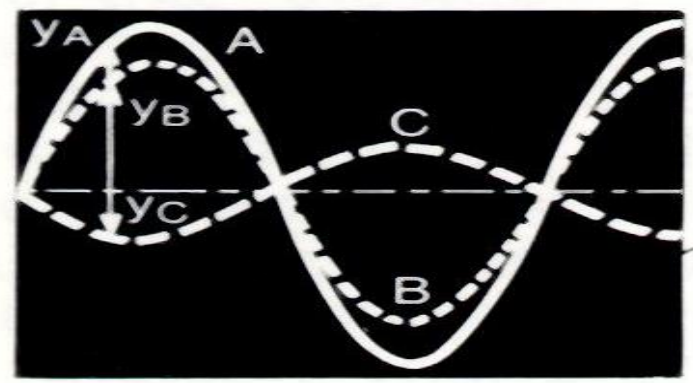
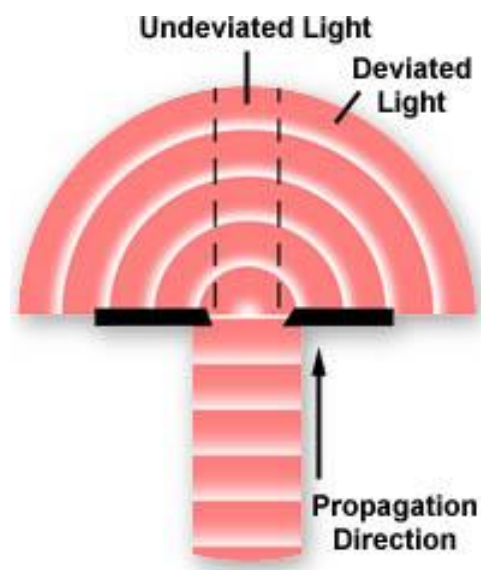
❖ 顯微鏡的成像

- 二次放大(物鏡, 目鏡)與中間影像(Intermediate Image)



❖ 影像的形成

- 光線在透過物體時，因物體的阻擋而產生二種情況：
 - Undeviated light (Direct light)* – 即為背景光亮。
 - Deviated light (Diffracted light)* – 因通過樣品而產生。
- } $1/2\lambda$ or 180 degree out of phase
- 二種光線產生破壞性的干擾 (destructive interference) 而觀察到物體的影像。
- ⇒ *Image = Direct light + Diffracted light*



YA = direct light
YC = diffracted light
YB = Image
YB = YA + YC



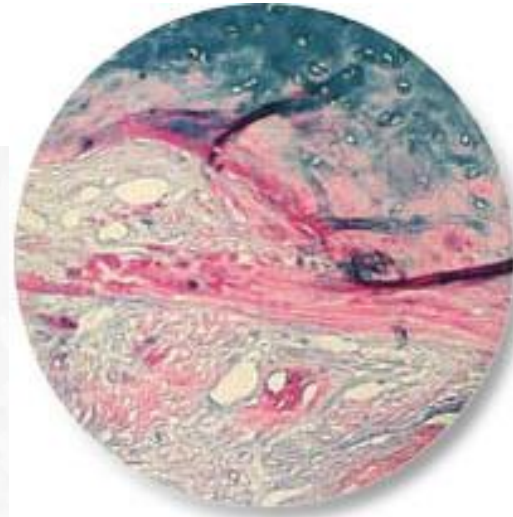
❖ Monochromatic aberration

Spherical aberration

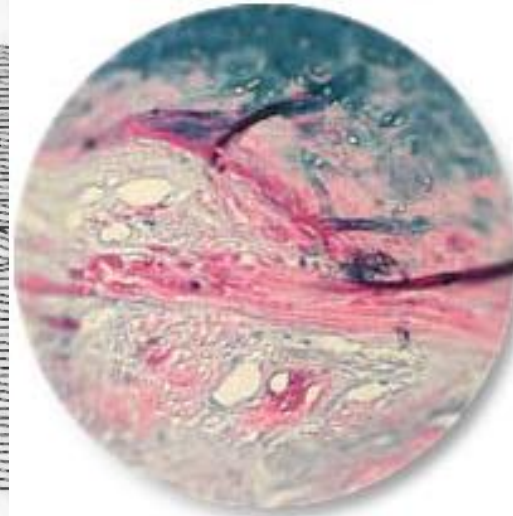
Coma

Astigmatism

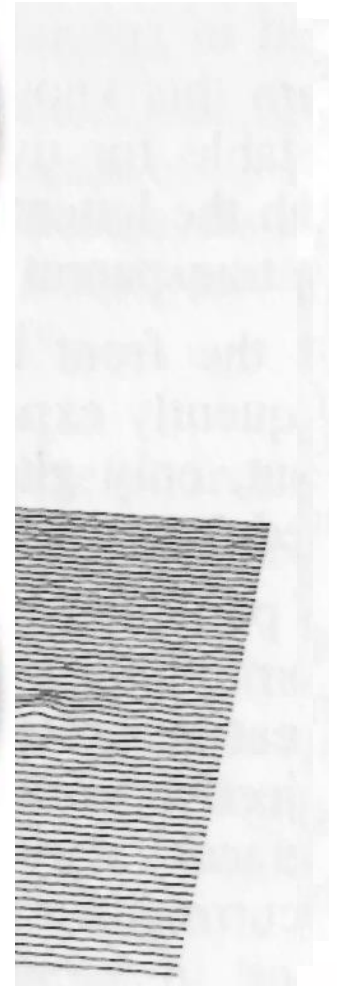
Image curvature



(b) Entire Viewfield in Focus



(c) Center in Focus

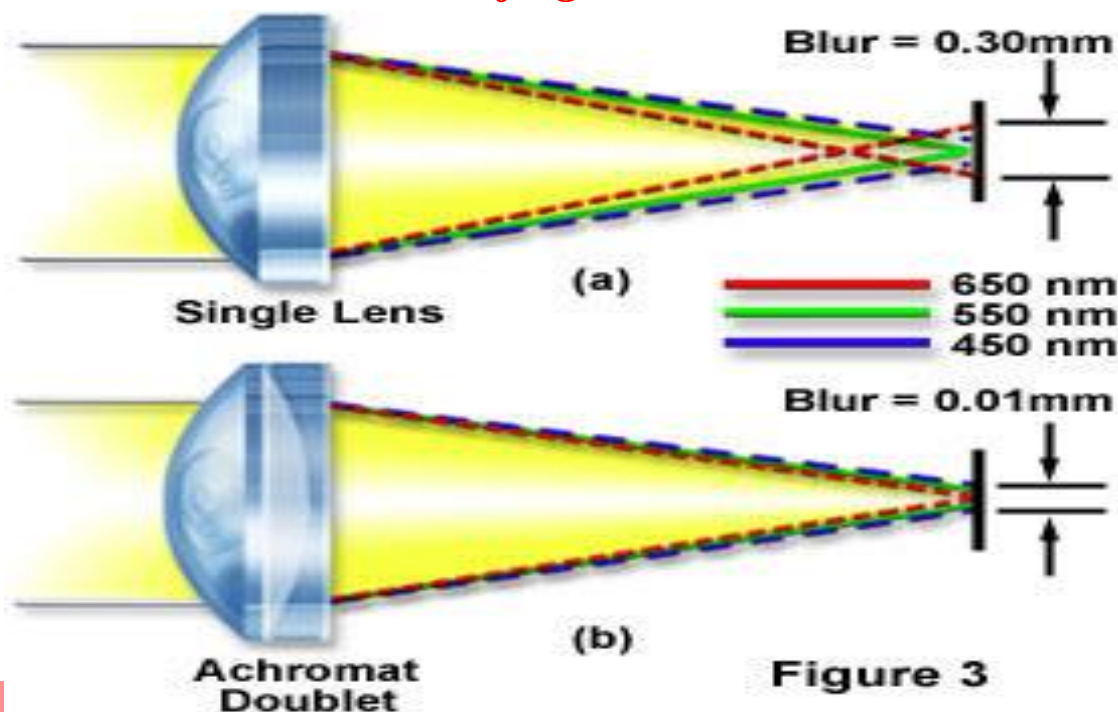


❖ Chromatic aberration

Image lenses are never free of aberrations, but most of these can be eliminated by a combination of lenses.

Axial chromatic aberration – colored fog

Lateral chromatic aberration – color fringes



❖ Objectives

- ❖ C PLAN: **Achromatic** objectives with a field performance up to **20 mm**.
Magnification: from 4x to 100x oil
- ❖ N PLAN: **Planachromatic** objectives with a field performance up to **22 mm**.
Magnification: from 2.5x to 100x oil
- ❖ PL FLUOTAR: **Semi-apochromats** with a field performance of at least **25 mm**.
Magnification: from 1.6x to 100x oil.
- ❖ PL APO: **Plan apochromats** with a field performance of up to **28 mm**,.
Magnification: from 10x to 100x oil, 150x and 250x are for semiconductor.
- ❖ Special Designed Objectives – HCX PL APO CS:
Some HCX PL APO objectives are re-calibrated for confocal system.

❖ How to “read” your objectives?

- $\infty/0.17/D$
 - ∞ : Infinite tube length objectives.
 - 0.17: 0.17mm蓋玻片 (0: Coverglass thickness 0; -: With or without coverglass; 0-2: for 0-2mm coverglass.)
 - A, B1, B2, C, D: DIC 稜鏡種類

- HC PL FLUOTAR

- 100x/1.40 OIL PH3
 - 100x: 物鏡倍率.
 - 1.40: 物鏡 NA 值
 - OIL: 油鏡
 - PH3: Phase Contrast
 - (CORR: Continuous setting to coverglass thickness)

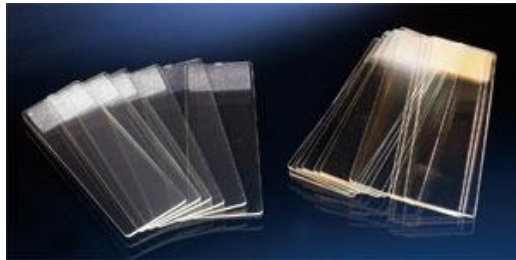




正立顯微鏡



倒立顯微鏡





Two kind of beam path

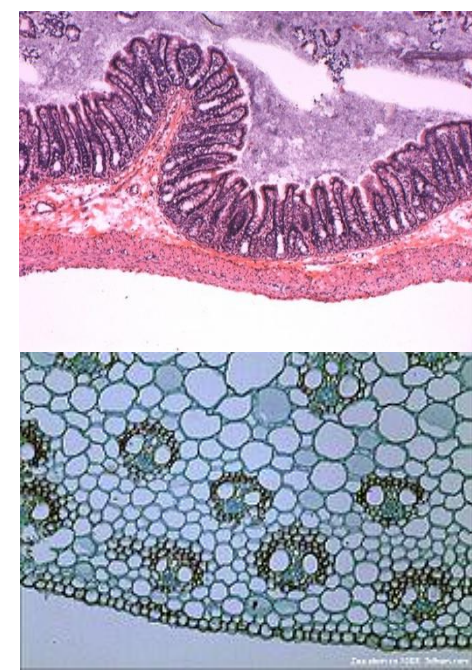
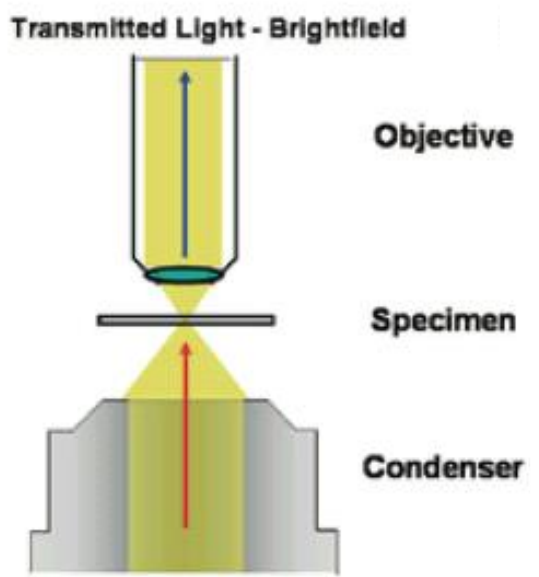
- Transmission Light
明視野 Bright Field
相位差 Phase Contrast

- Incident Light
螢光 Fluorescent



- Bright-Field Microscopy -

- Bright Field - 適用於已染色、強對比的樣本。





- Phase Contrast Microscopy -



為什麼要有Phase Contrast?

❖ 觀察的限制

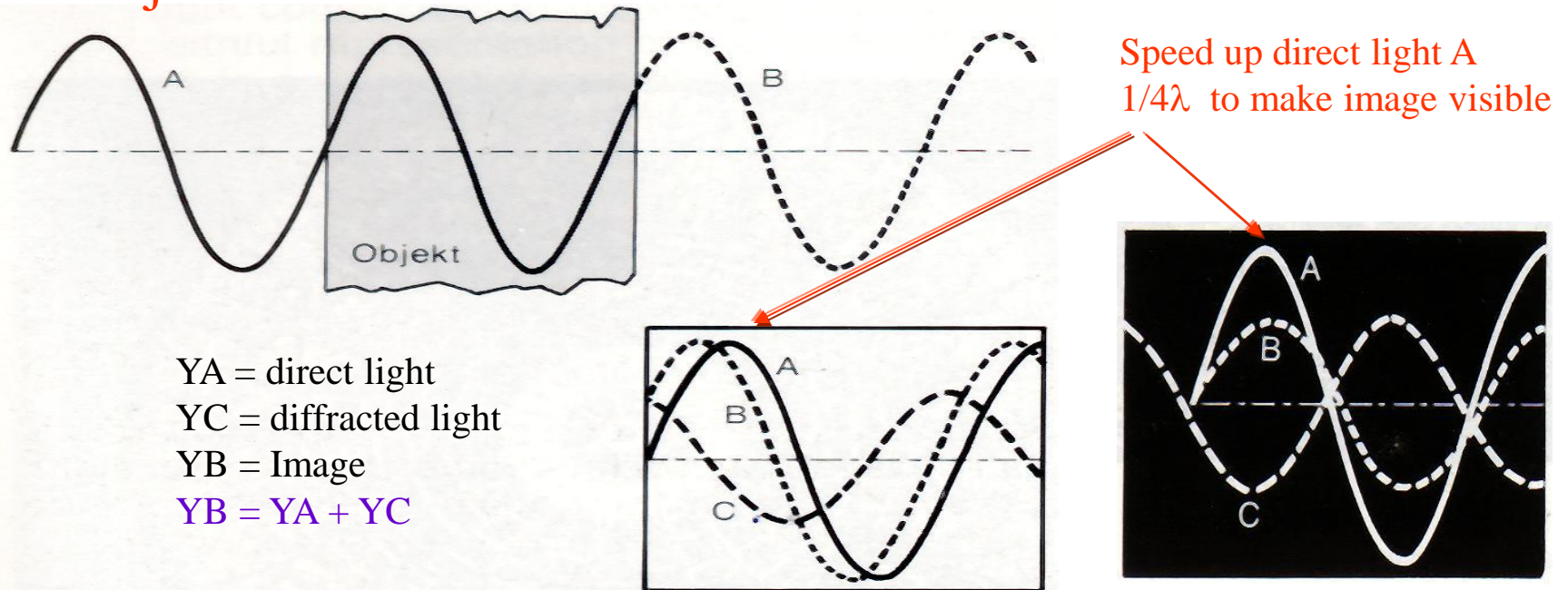
- 受觀測物體可區分為：**Amplitude objects** – 已染色或吸光率高的高對比樣品。
Phase objects – 未染色之活細胞。
- 人的眼睛只能觀察到：
 1. 顏色的變化 – 波長wavelength.
 2. 光強度的改變 – 振幅wave amplitude.
- 未染色之活細胞通常不能吸收光線，他們只是改變入射光線約 $1/4\lambda$ 的位相差，而這樣變化人眼是幾乎無法察覺的.....
⇒我們只能觀察Amplitude objects 而無法看到Phase objects。

❖ 解決的方法

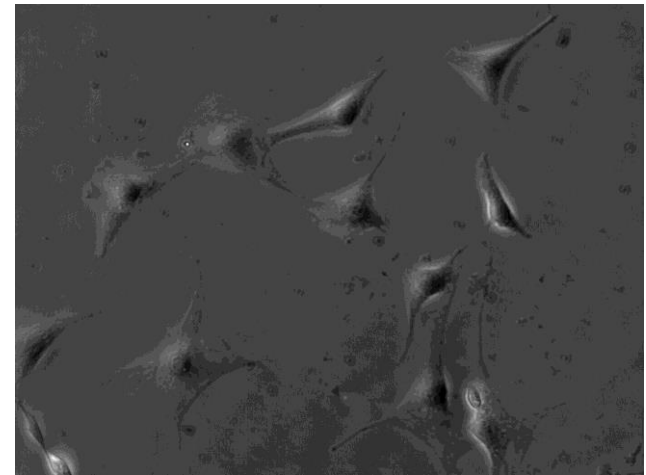
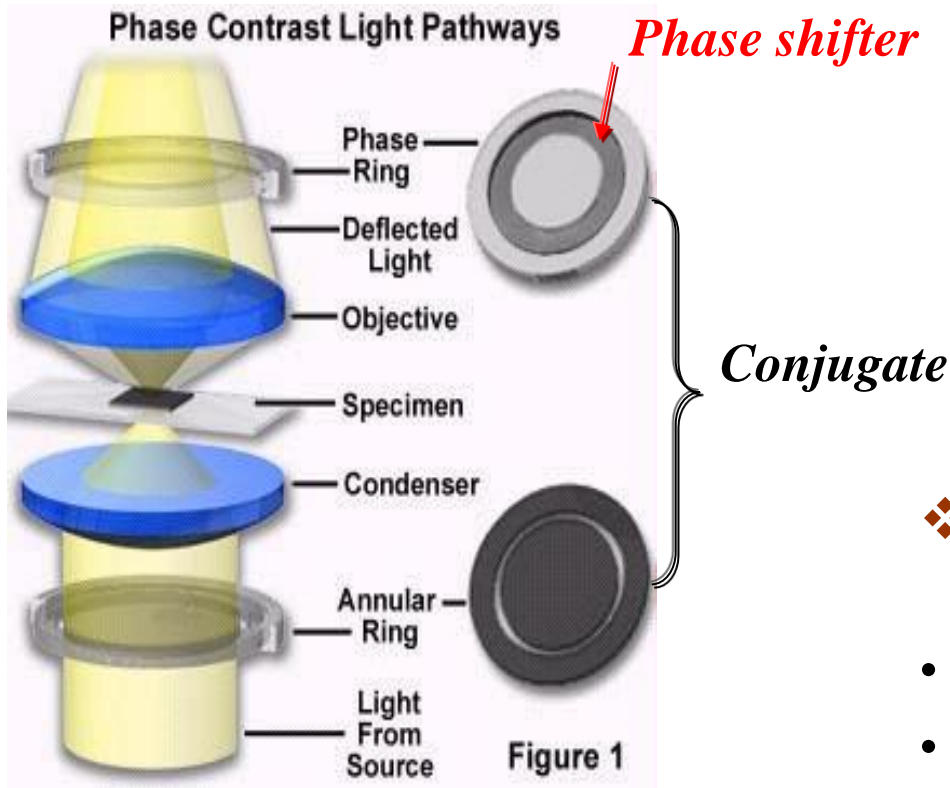
- 將原本只有phase變化的物體，藉由顯微技術的設計，將其轉換為人眼可觀察的amplitude變化，此即為**Phase Contrast Microscopy**.
- 1930年代，Fritz Zernike成功的將phase objects的影像以amplitude方式展示出來而可被人眼容易的觀察，奠定了Phase contrast microscopy的基礎，Zernike也因此於1953年獲得諾貝爾物理獎。



Phase object

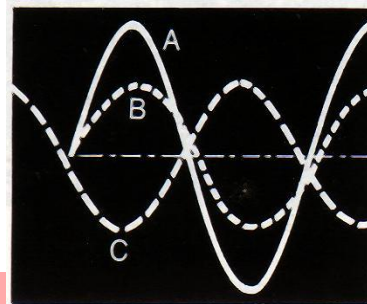
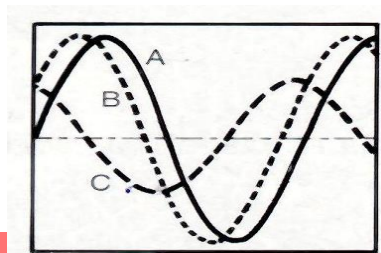


- 依據Abbe的成像理論，物體影像的產生是由direct light (0th order)與diffracted light干擾加成而產生。
- 因此，direct light與diffracted light呈現180度 ($1/2\lambda$) 的位相差時，物體影像對比將最好。
- 在Phase的樣本中，藉由加快或減慢direct light $1/4\lambda$ ，使phase的樣本轉換為amplitude的形式而可被觀察。
- 加快 $1/4\lambda \Rightarrow$ Positive or dark phase contrast。 減慢 $1/4\lambda \Rightarrow$ negative or bright phase contrast。



❖ *Phase Contrast Microscopy* *應用上的限制*

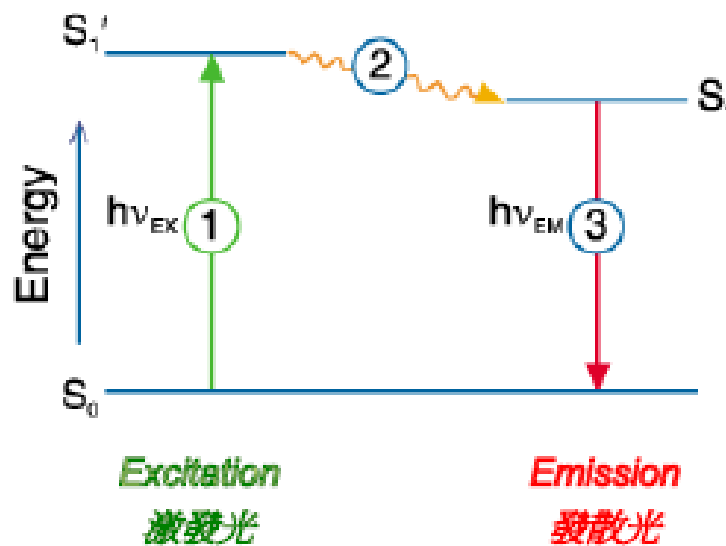
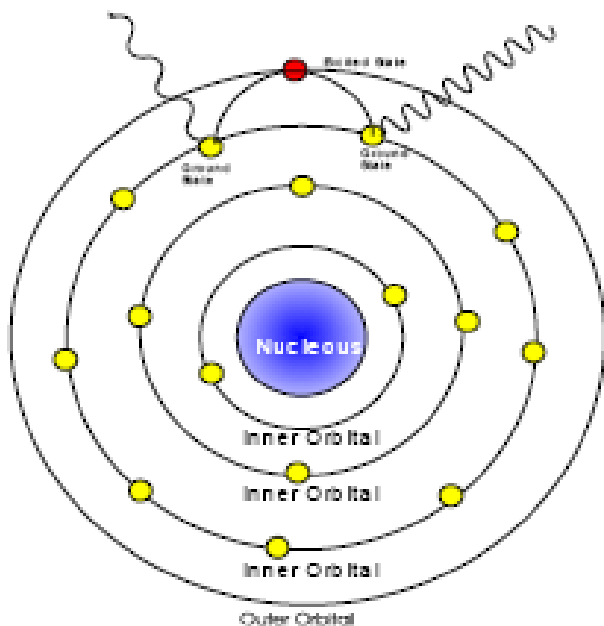
- 解析度限制。
- Phase影像周圍的光暈現象。
- 不適合用於過厚的樣品觀察。
- 使用時應加入綠光濾片。



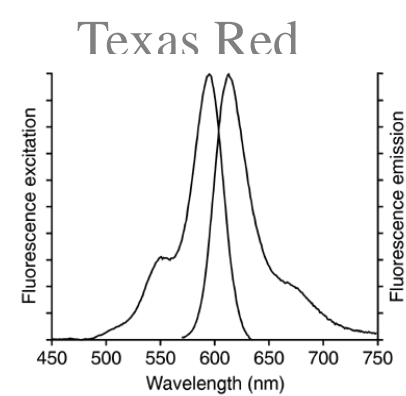
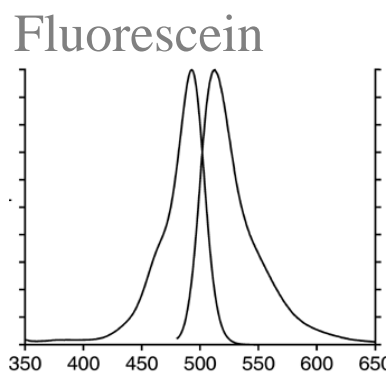
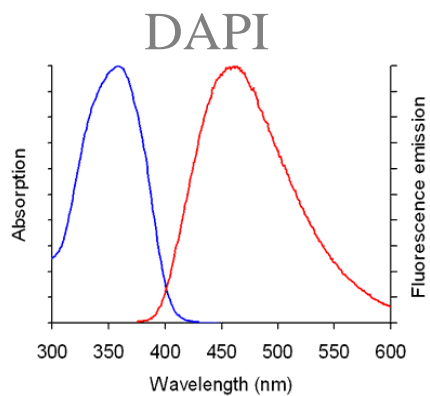
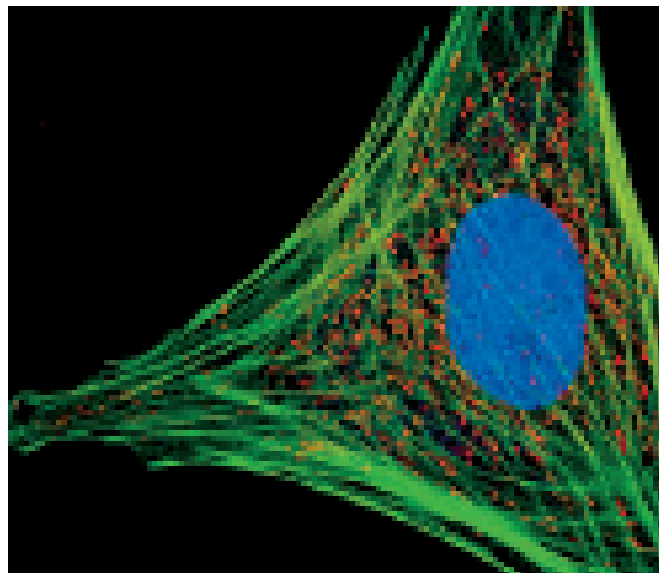
什麼是螢光？

- Incident Light

Fluorescence



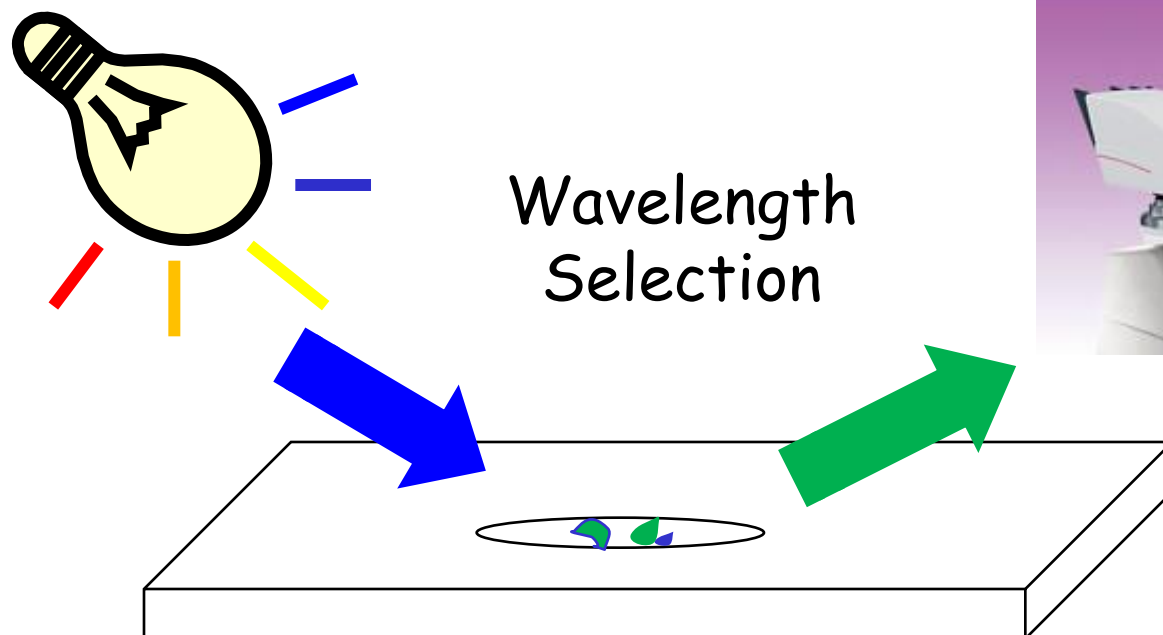
- Fluorescence 螢光



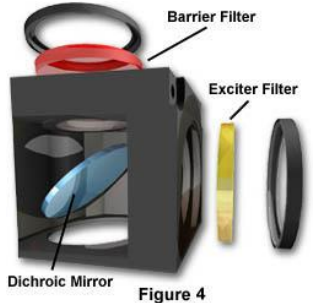
- Fluorescence 螢光

Illumination System
(Excitation)

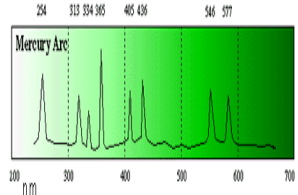
Detection System
(Emission)



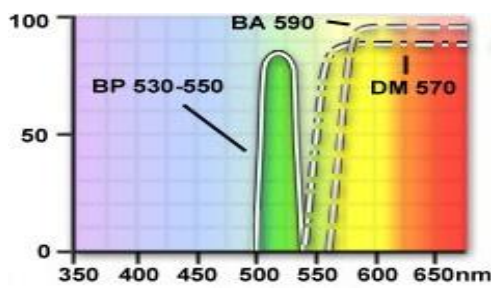
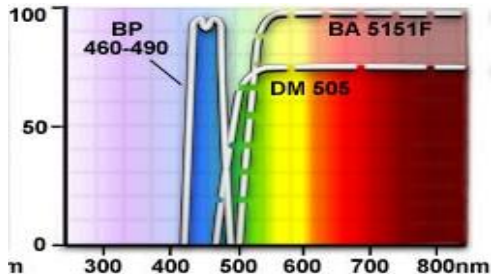
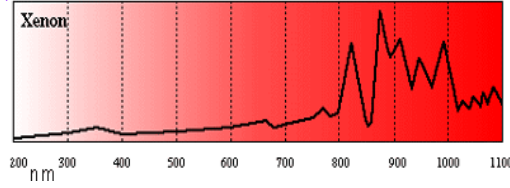
- Conventional Fluorescence Microscope



HBO

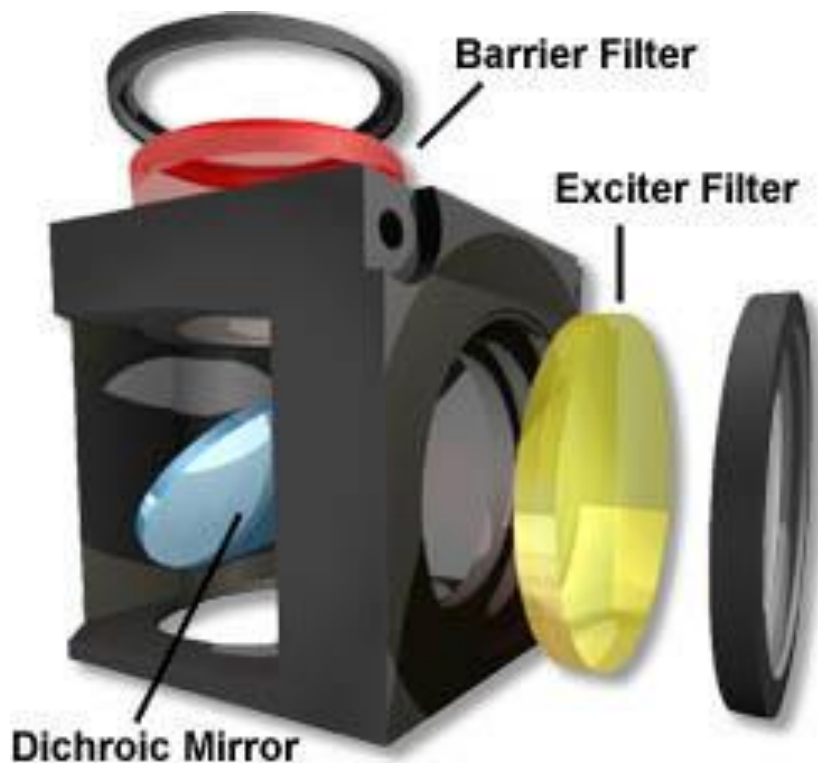


XBO



- Conventional Fluorescence Microscope

Wavelength Selection -- Filter

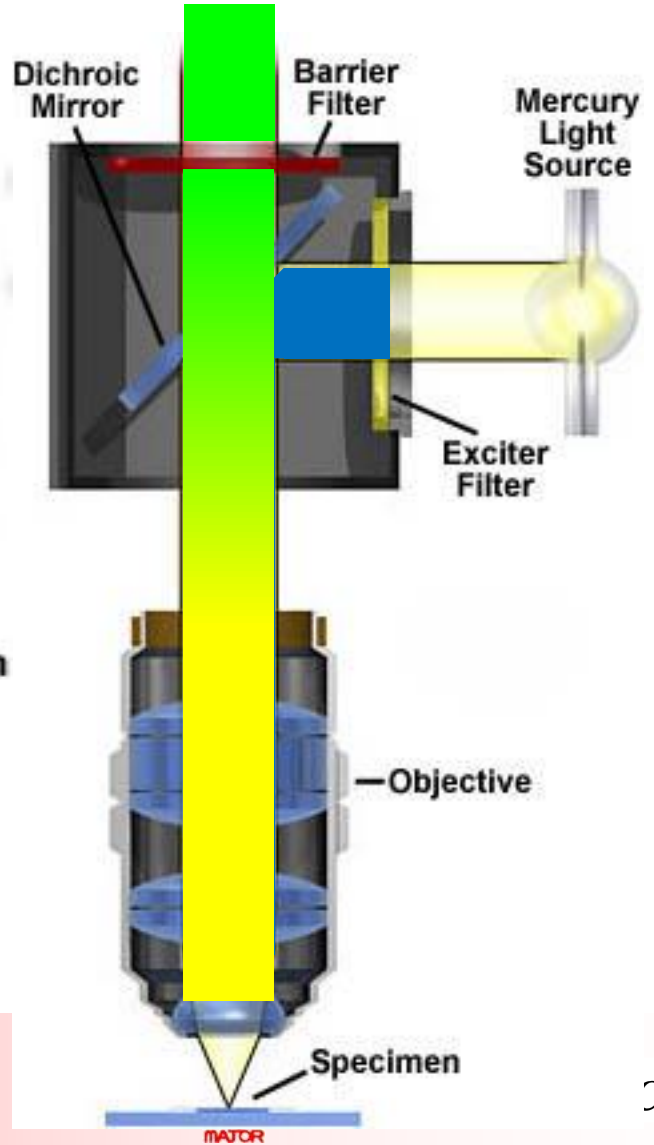
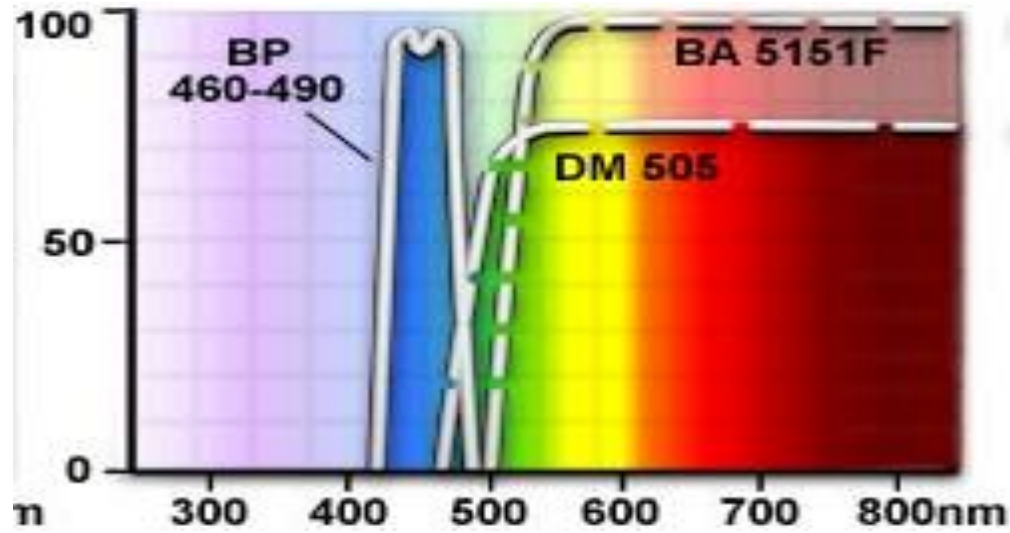


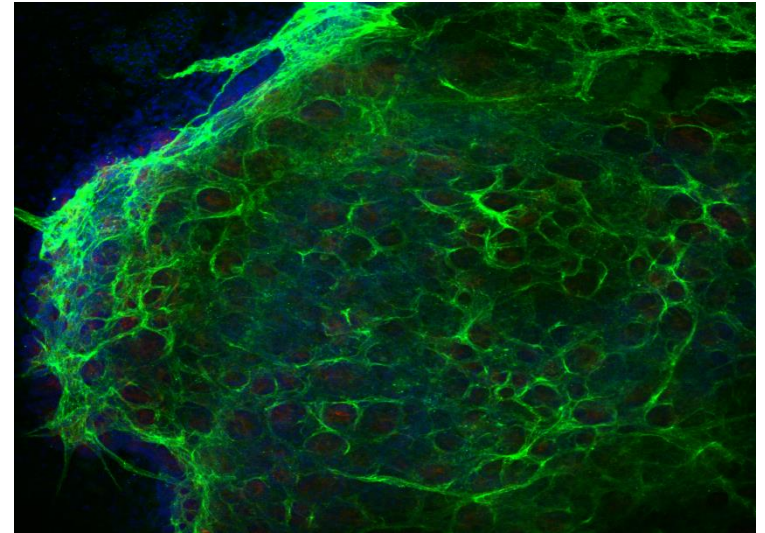
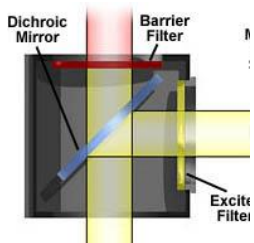
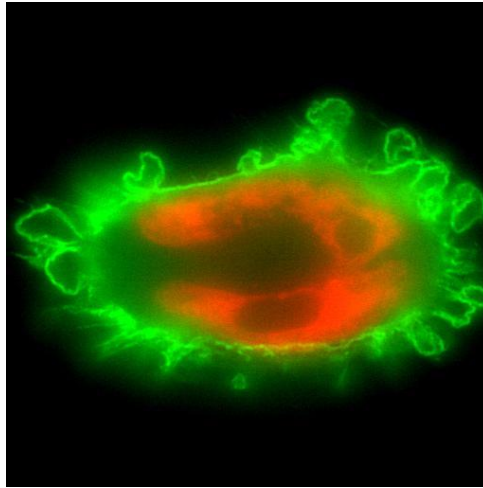
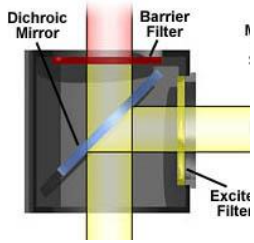
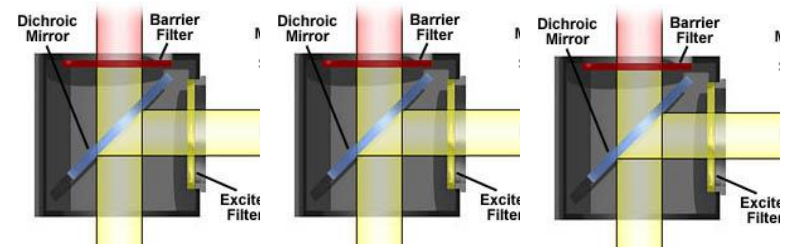
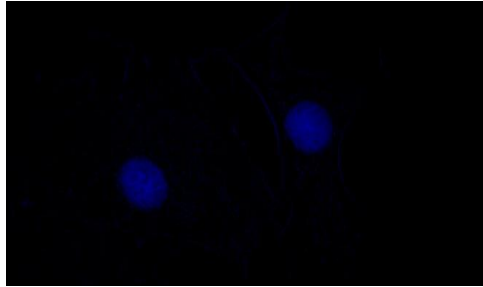
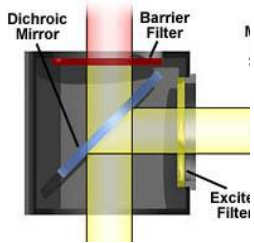
Short Pass

Long Pass

Band Pass

- Conventional Fluorescence Microscope





Incubation

溫度需維持在**35-37°C**。

- 大部分哺乳動物細胞需生長於35-37°C
 - 溫度控制不良：
 - 代謝失調
 - 溫度過低可能造成生長遲緩
 - 溫度過高會造成 **heat-shock** 或死亡
- 38.5-39.5°C 數小時即會死亡



溫度
Temperature

pH

濕度
Humidity

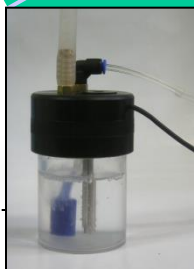
需維持**5%CO₂**

- 緩衝系統 CO₂/HCO₃⁻
- 細胞培養適宜環境 **pH 7.4**.
- pH 低於6.8常會抑制生長



需保持適當濕度

- 培養液蒸發
 - 細胞受損或死亡
 - 培養液濃度改變 影響細胞生長





顯微鏡上細胞影像觀察





+



MICROSYSTEMS

=

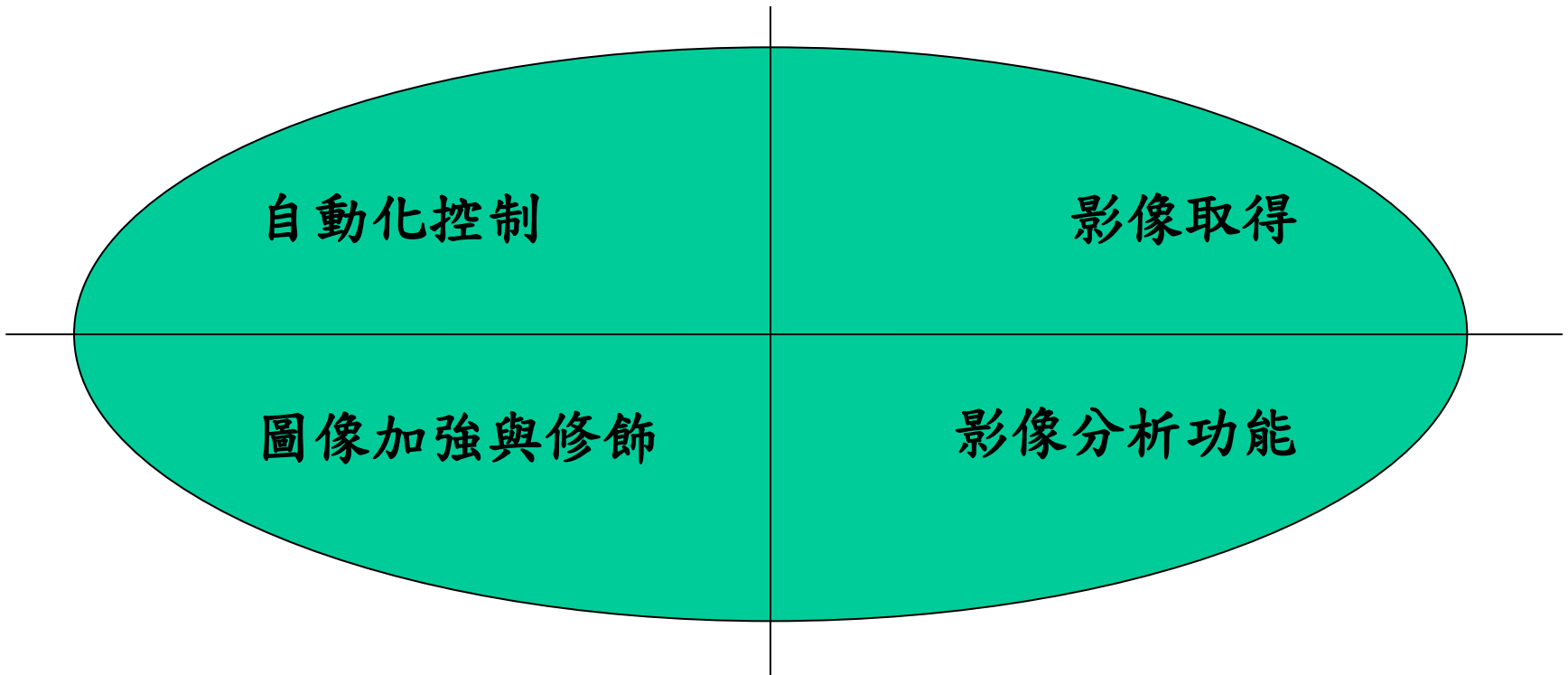
Reach High !



Major Instruments Co., Ltd.



MetaMorph[®] imaging system

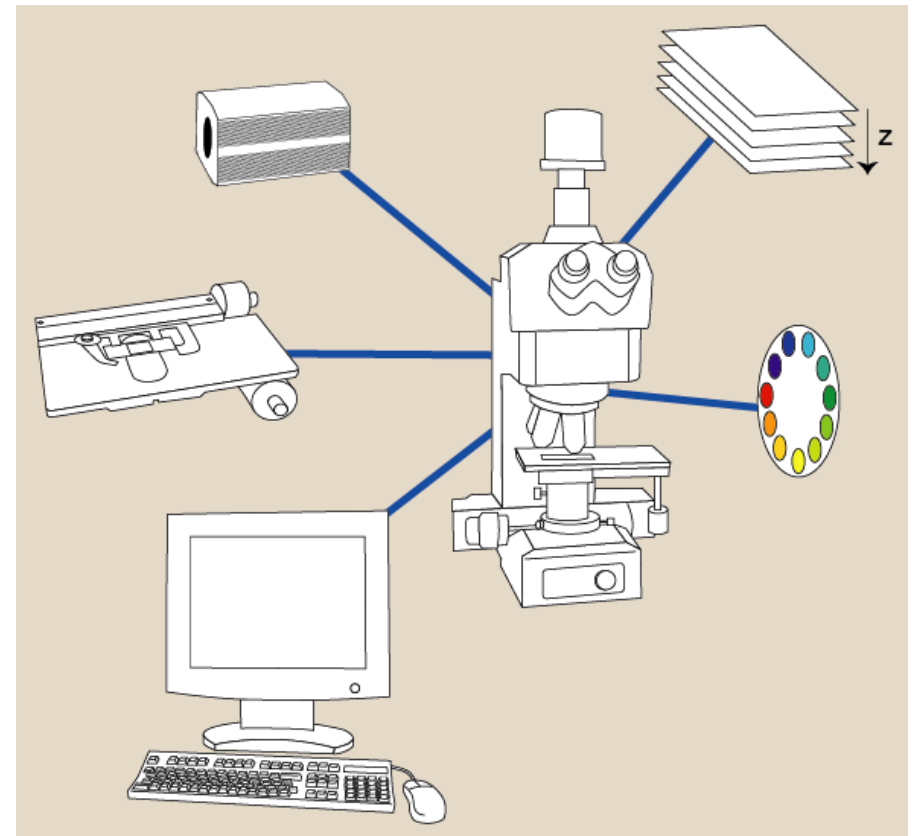


Major Instruments Co. Ltd

Reach High!

自動化控制

- 顯微鏡系統
- 影像CCD
- 電動XYZ三軸控制
- 光源選擇
- 共軛焦模組
- TTL訊號





自動化控制

The Leica logo, consisting of the word 'Leica' in a red, cursive script font.

MICROSYSTEMS



OLYMPUS



The Leica Microsystems logo, with 'Leica' in red script and 'MICROSYSTEMS' in yellow sans-serif below it.



HAMAMATSU



....and etc.



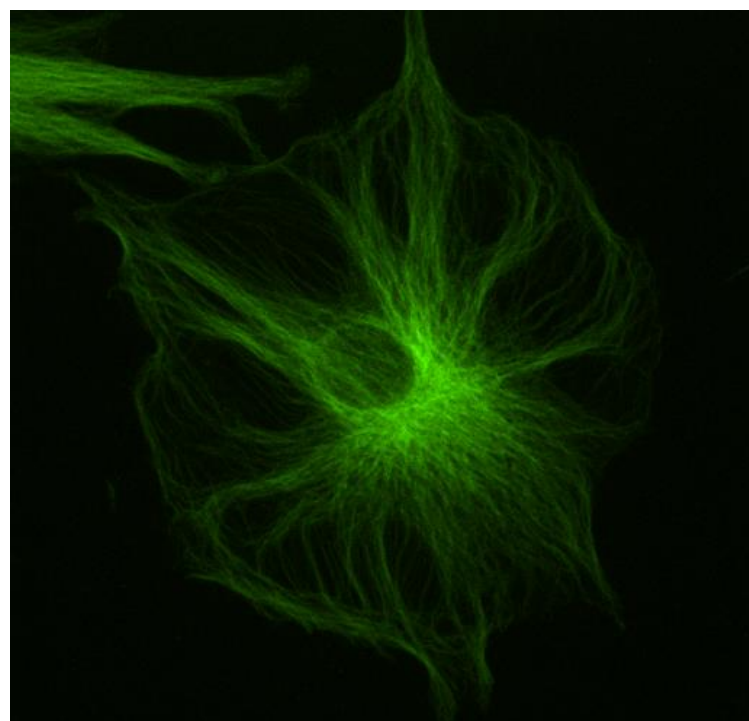
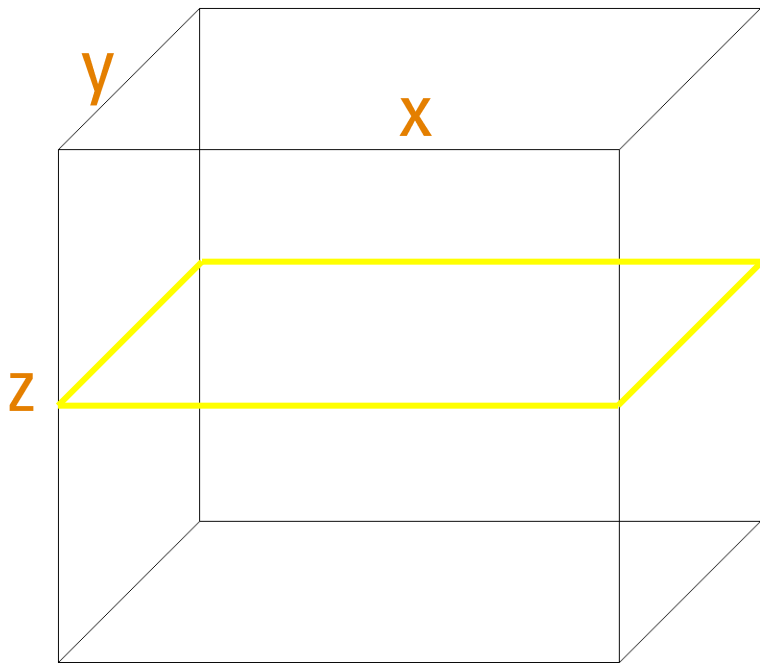
Major Instruments Co., Ltd

Reach High !

影像取得

- 單一圖層擷取(XY)
- Z軸序列擷取(XYZ)
- 時間序列擷取(XYT)
- 多波長擷取(XYλ)
- 位置移動擷取(XYP)
- 多維合併序列擷取

一般平面



Illum: FITC Mag: 63X X: 0.00 Y: 0.00 Z: 0.00

*Live (100%)

Acquire

Acquire Image: Acquired

Save Live! Save to: C:\MMM...Acquired001.tif Set Save...

Save w/Sequence

Exposure Time: 100 ms AutoExpose

Binning: 1

Camera Area: -> Full Chip

Center Quad.

Use Active Region

Show Live

Live Bin: 1

Temp: -20.8 c

Setting [Modified]:

Close Less << Setting: Load ... Save Save As...

Display Acquire Correct Annotate Special Live Replay

Image Scaling:

Lo % 0 Hi % 0

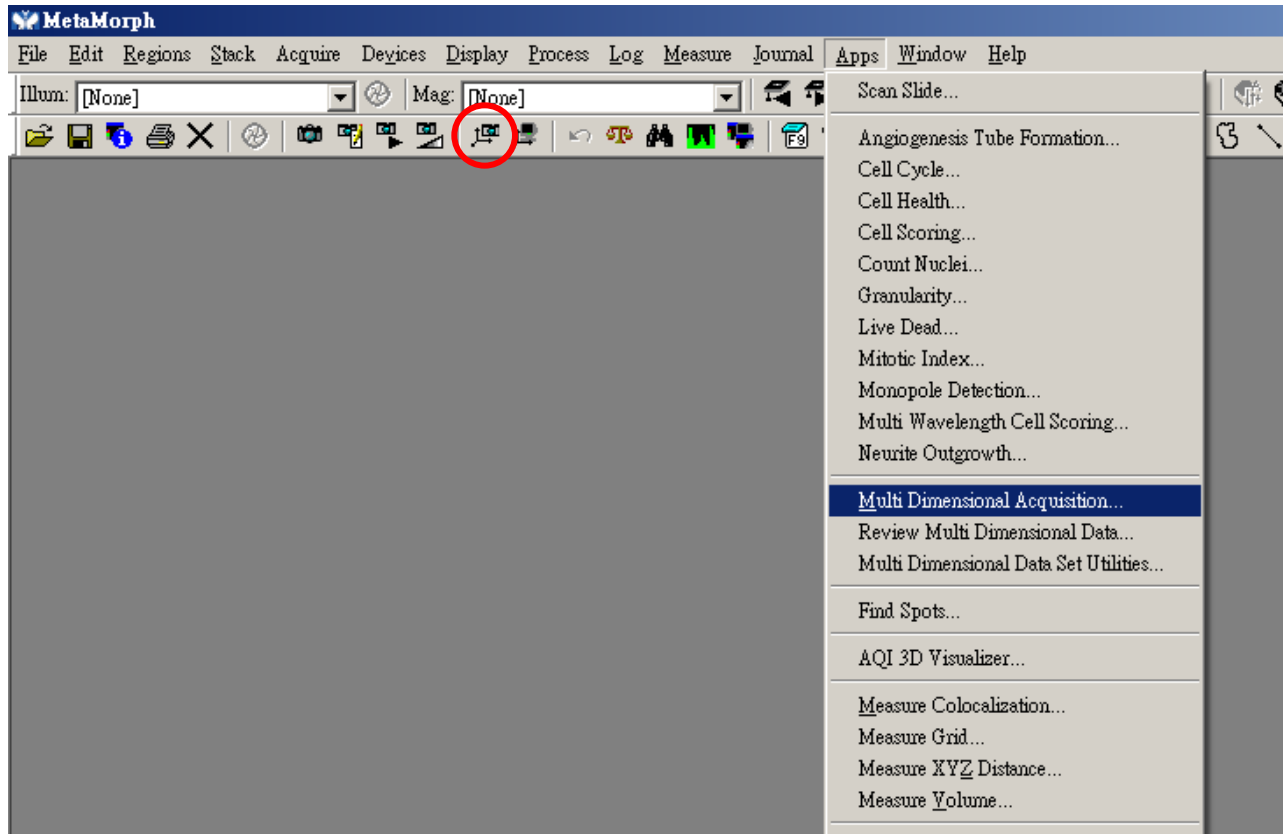
Autoscale

Scale within the active region

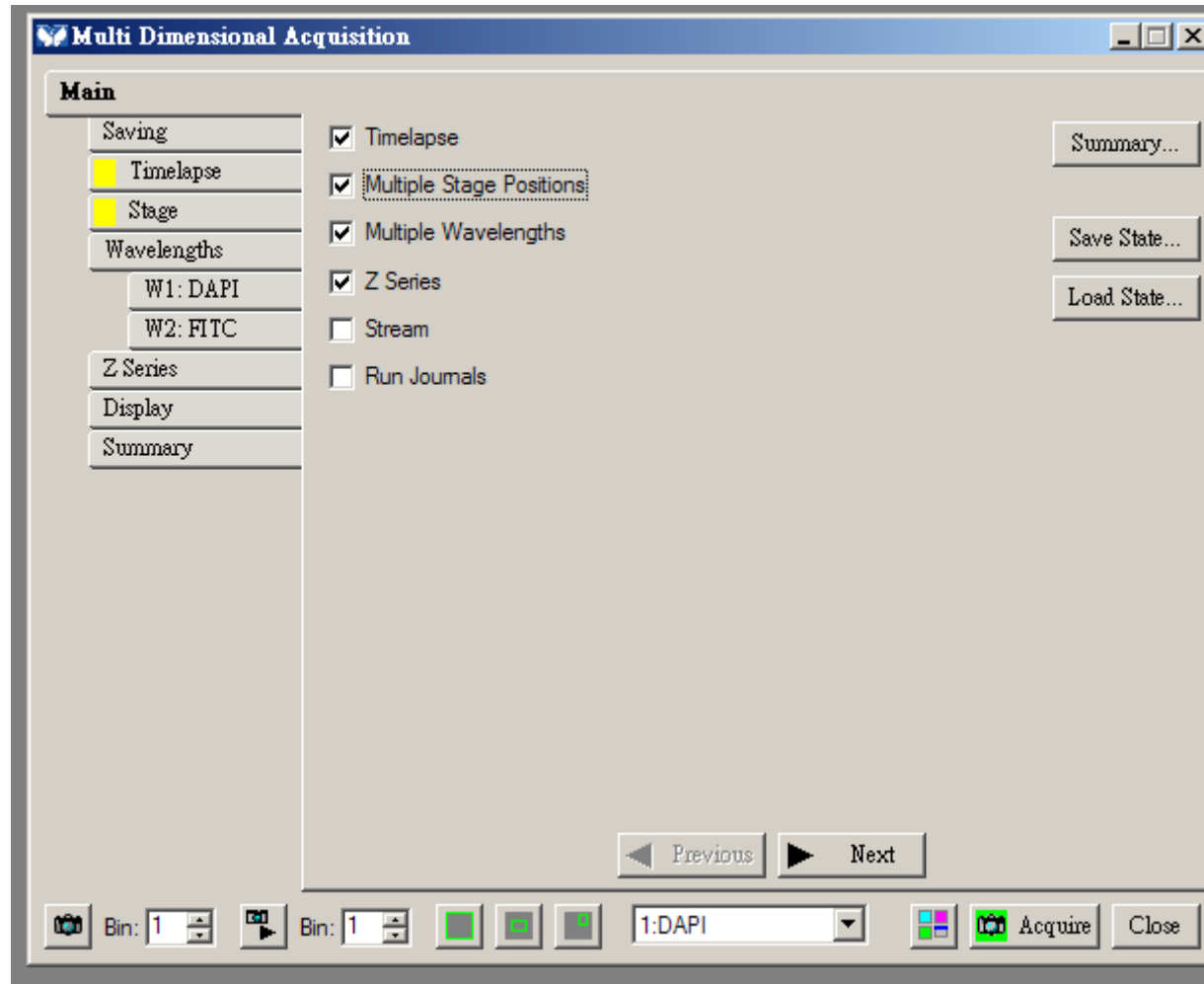
Image Gamma: 1.00 $\gamma=1$

Reset Display

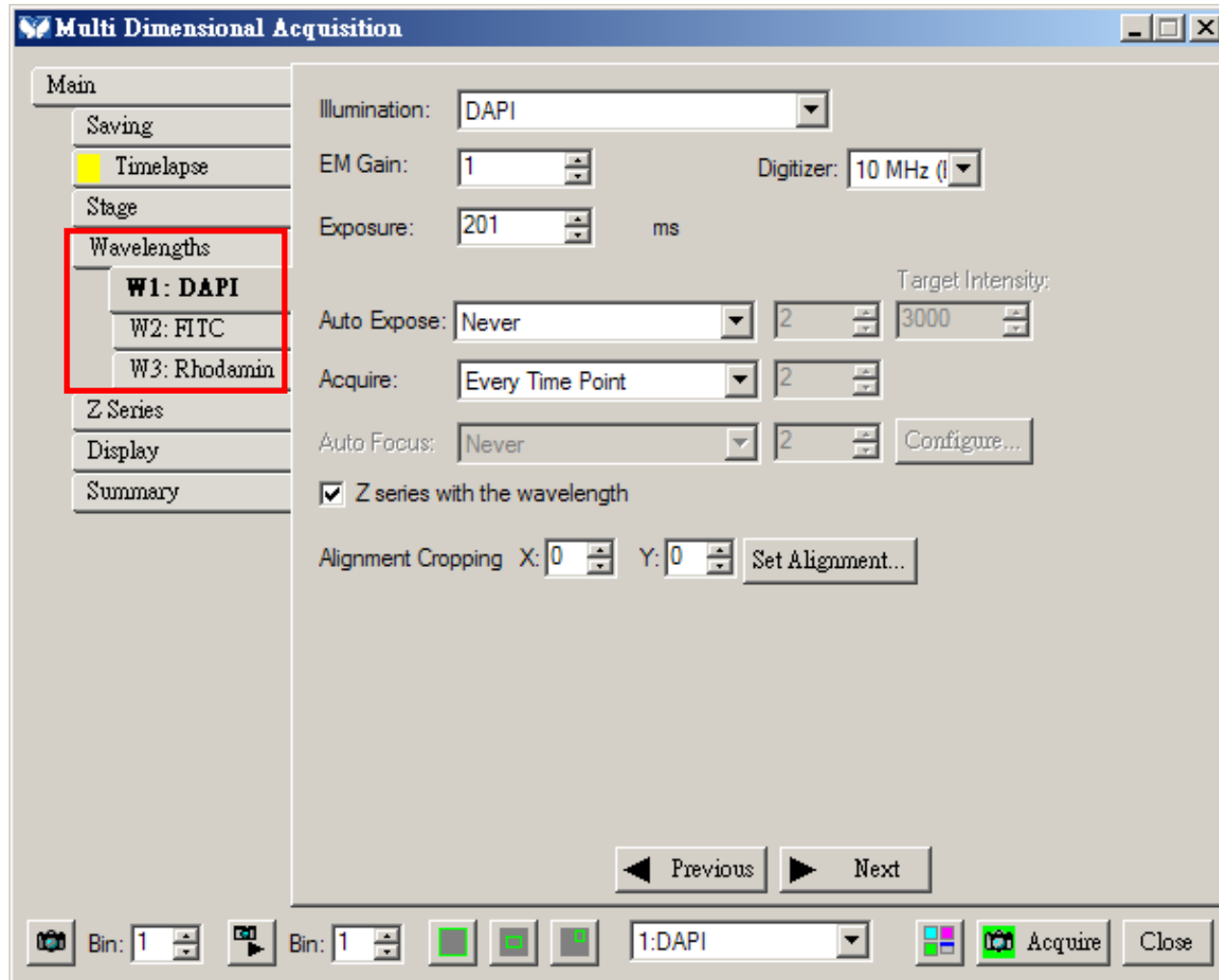
多維影像拍攝 Multi Dimensional Acquisition



Multi Dimensional Acquisition



多維影像攝影---多重螢光





MetaMorph®



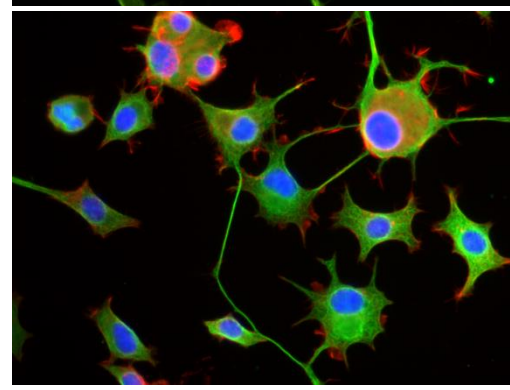
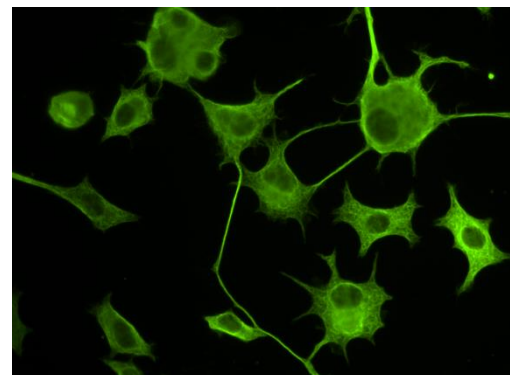
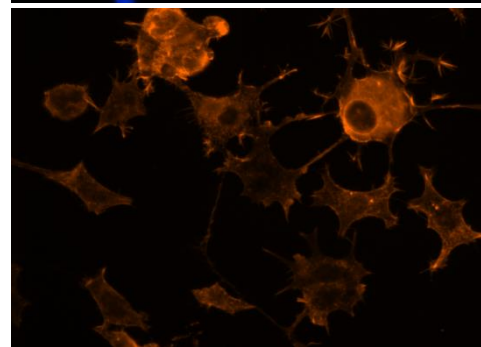
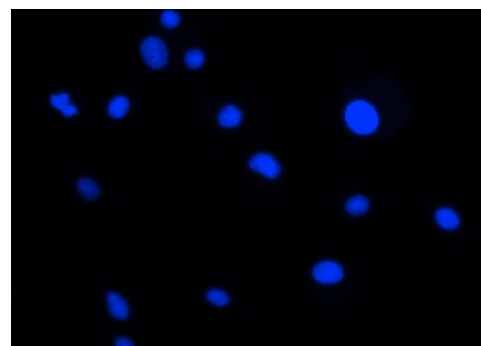
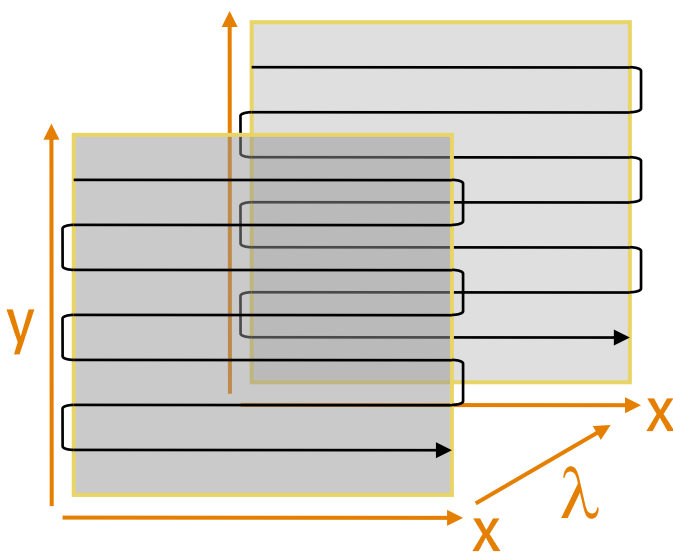
MetaFluor®



MetaVue™



MetaMorph®



Multi Dimensional Acquisition

Main

- Saving
- Timelapse**
- Stage
- Wavelengths
 - W1: DAPI
 - W2: FITC
- Z Series
- Display
- Summary

Experiment Length

Number of time points: 5

Duration: 4 seconds

Time Interval: 1 seconds

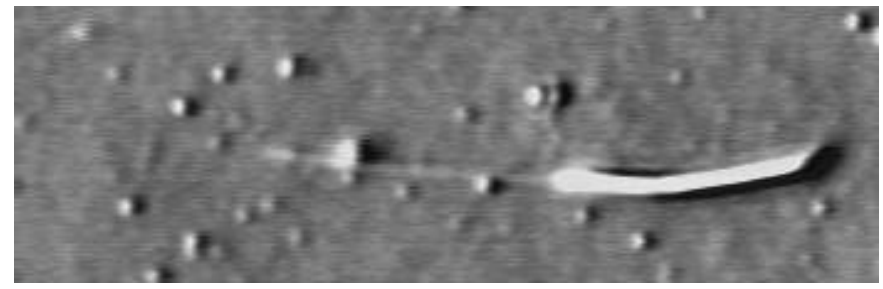
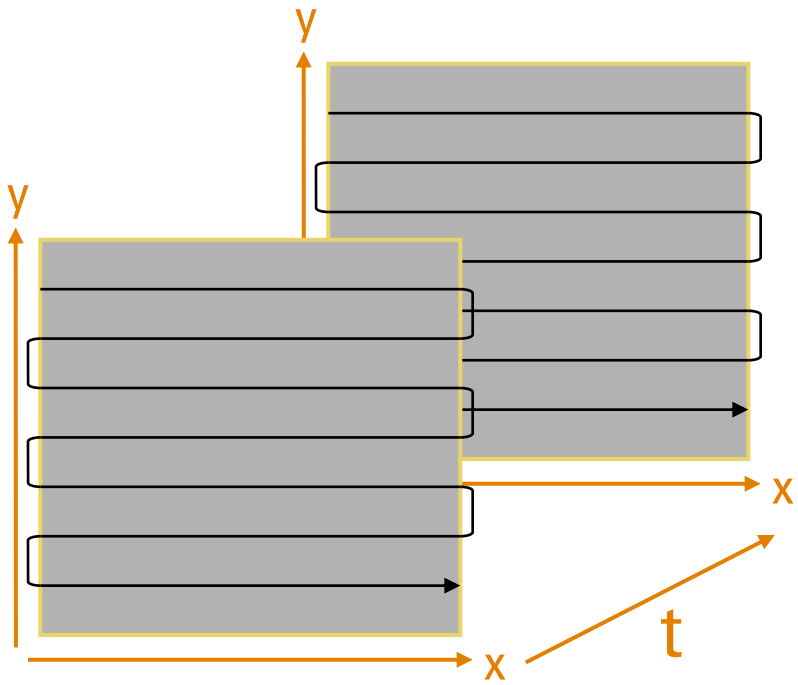
Estimated minimum interval: 1.81 sec

Interval specified is below calculated minimum

Previous Next

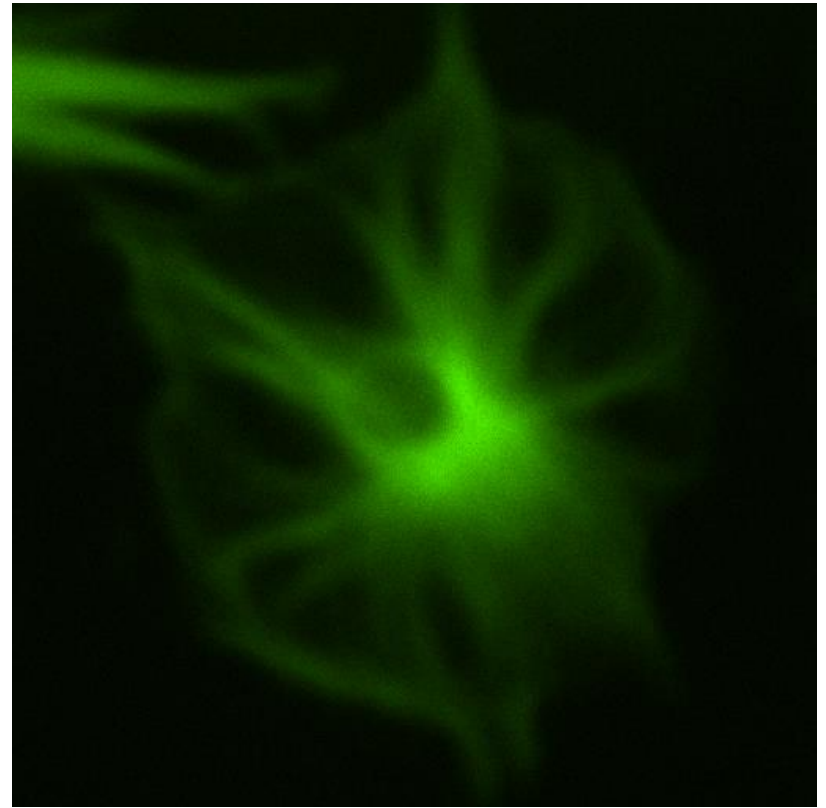
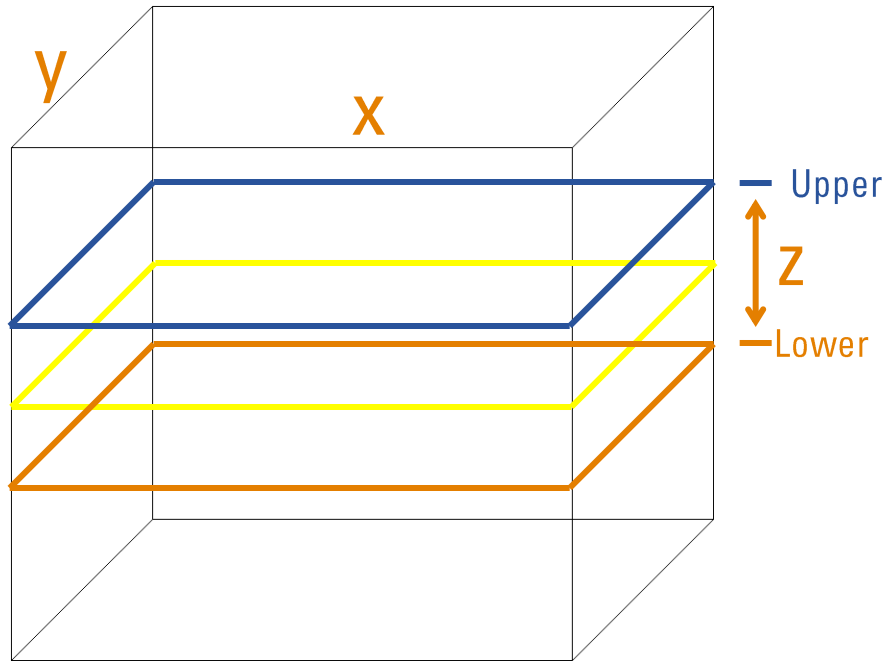
Bin: 1 Bin: 1 1:DAPI Acquire Close

多維影像攝影---時間序列

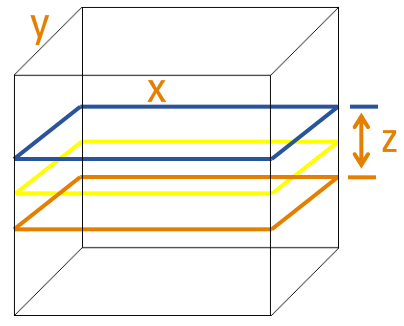


Microtubule based motility

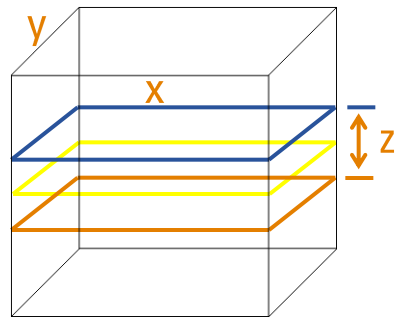
多維影像攝影--- Z軸拍攝



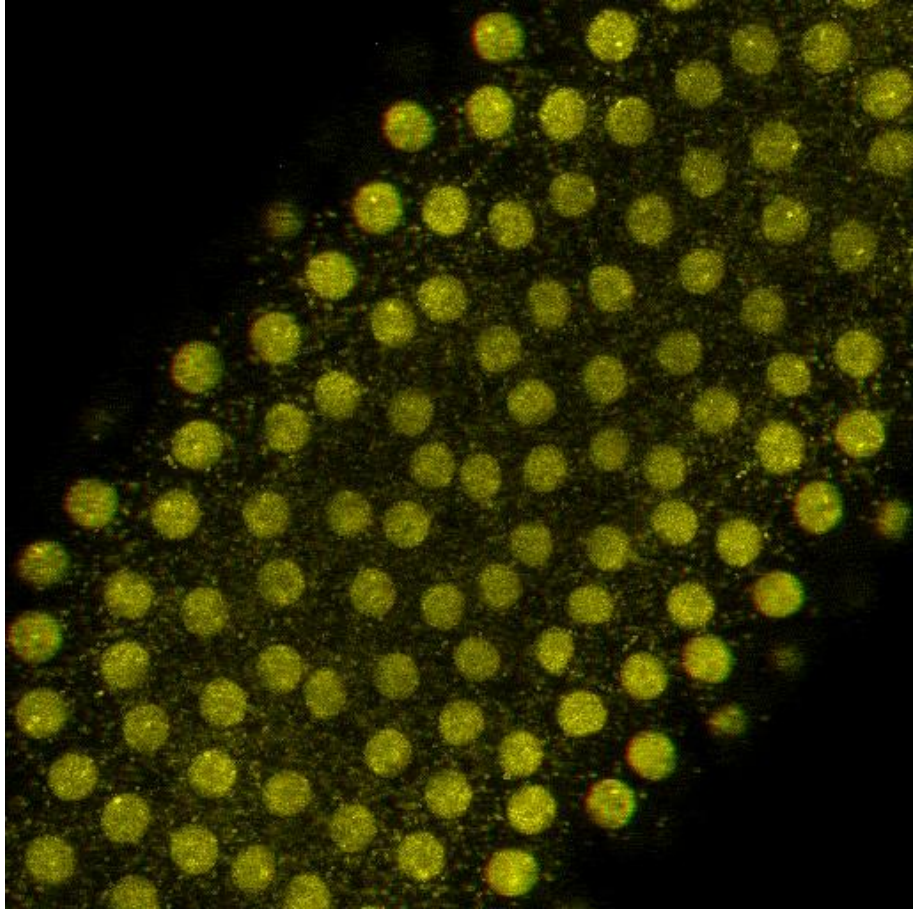
多維影像攝影---Z軸時間序列



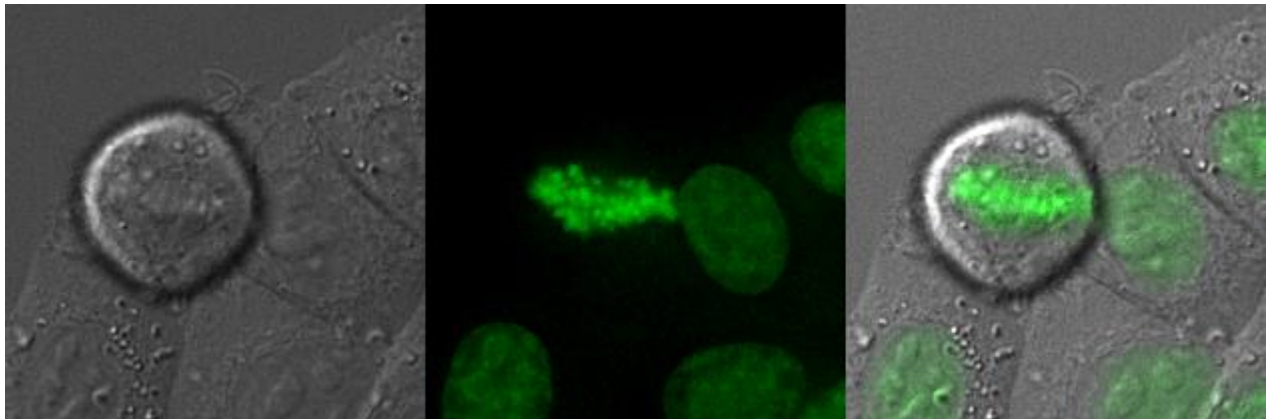
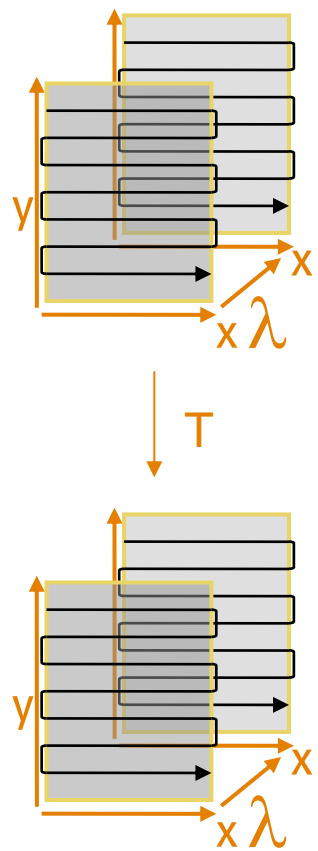
T



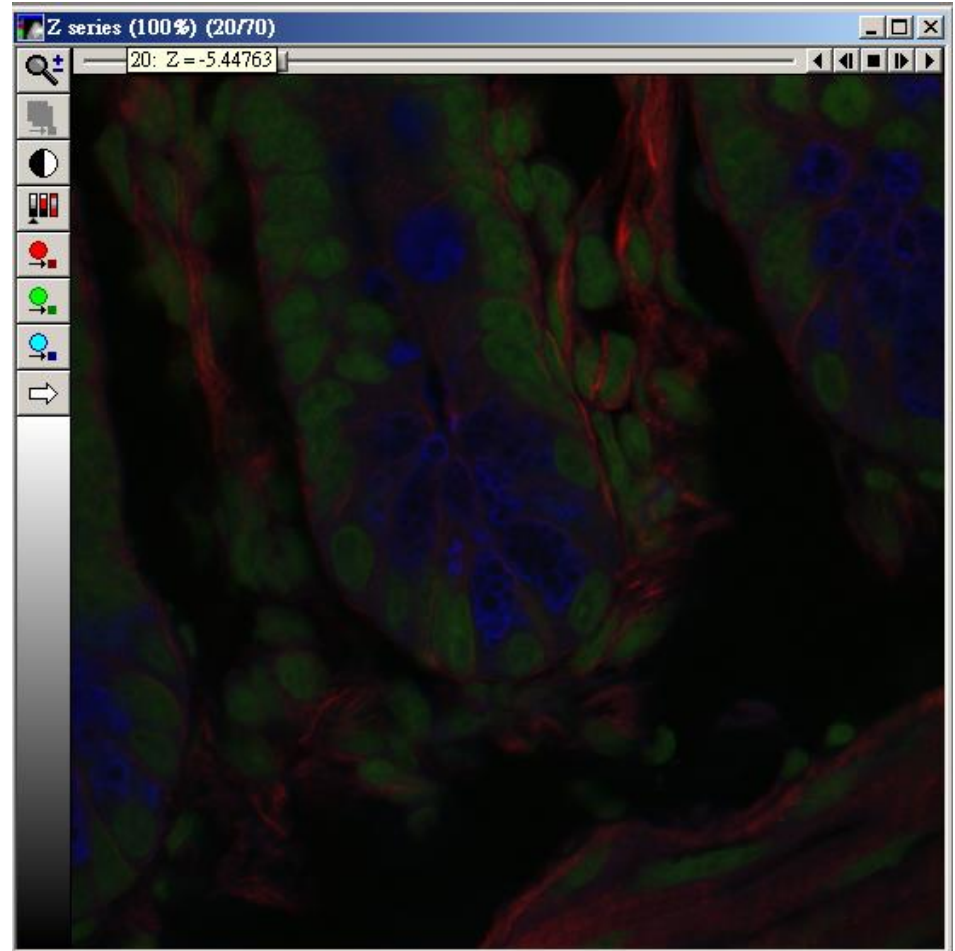
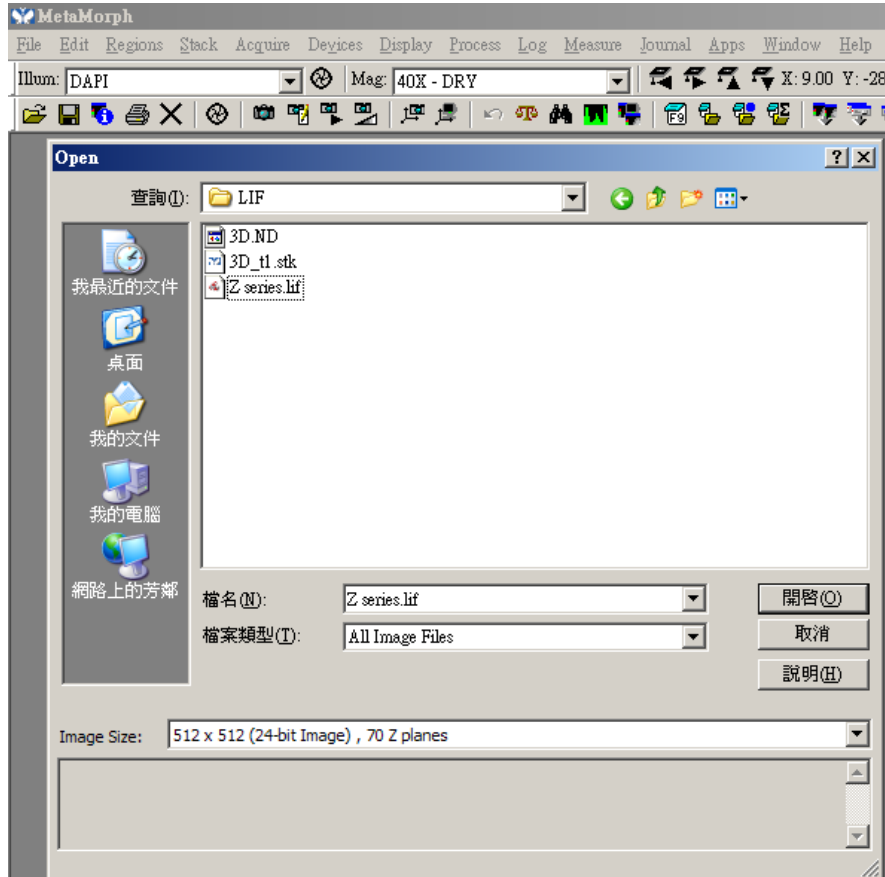
T



多維影像攝影---多重螢光時間序列

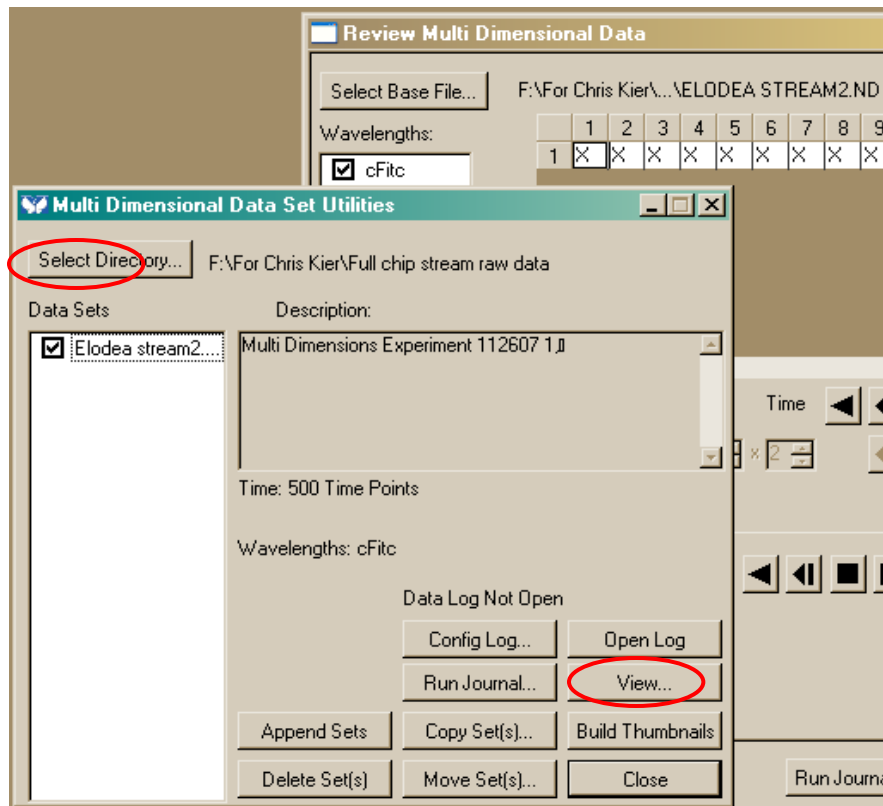


File → Open (.tif, jpg, bmp,)

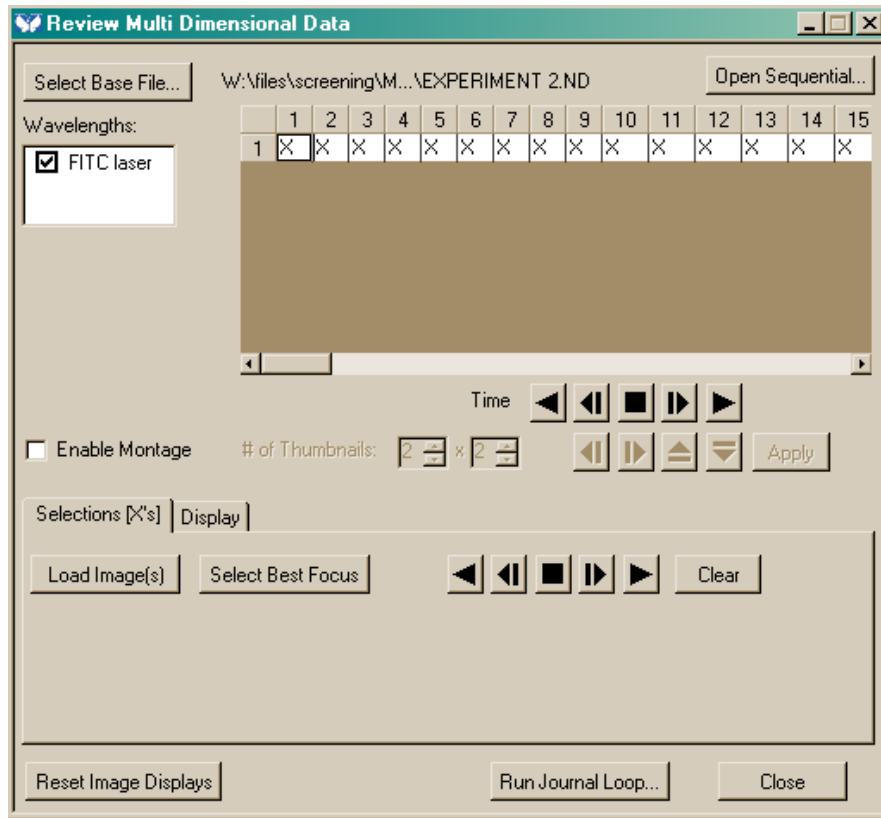


Apps → Review Multidimensional Data

使用於觀看,輸出,分析,在MetaMorph Multi Dimensional Data 具有(*.nd)的影像序列檔案

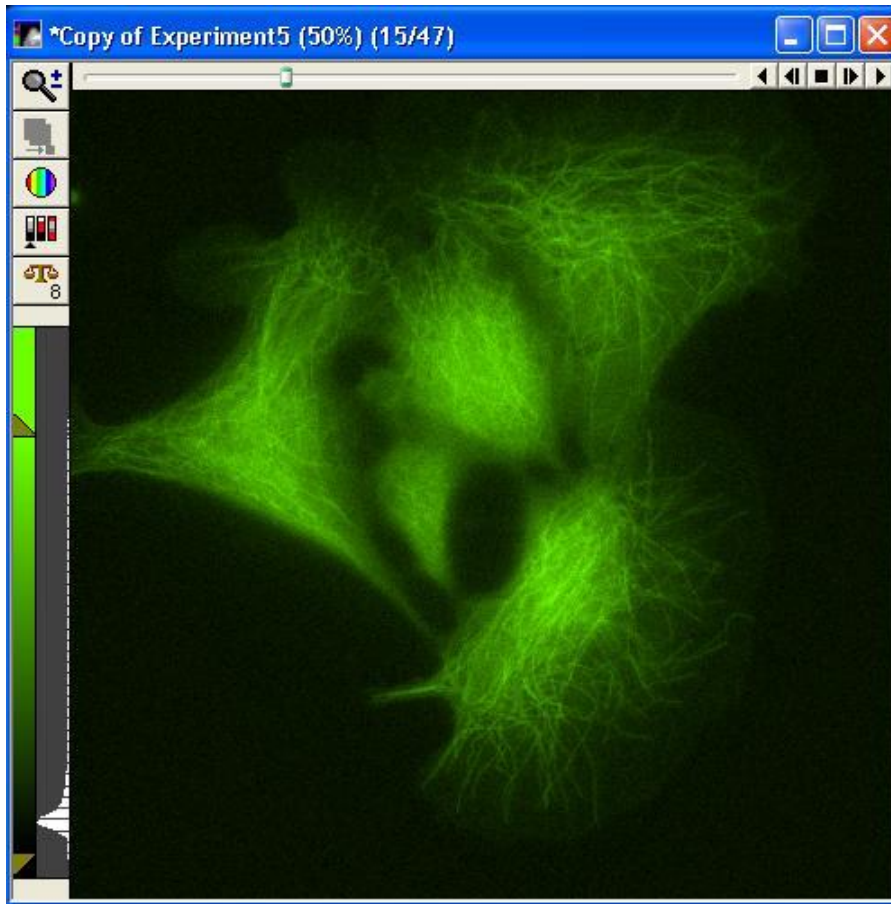


Review Multidimensional Data



- Load Image
勾選要觀察的波長與時間,Z軸的影像序列,按下Load可整個輸出
- Select Best Focus
在拍攝多時間與Z軸影像的檔案中,可用此選項幫助選出最佳聚焦平面.

Stacks

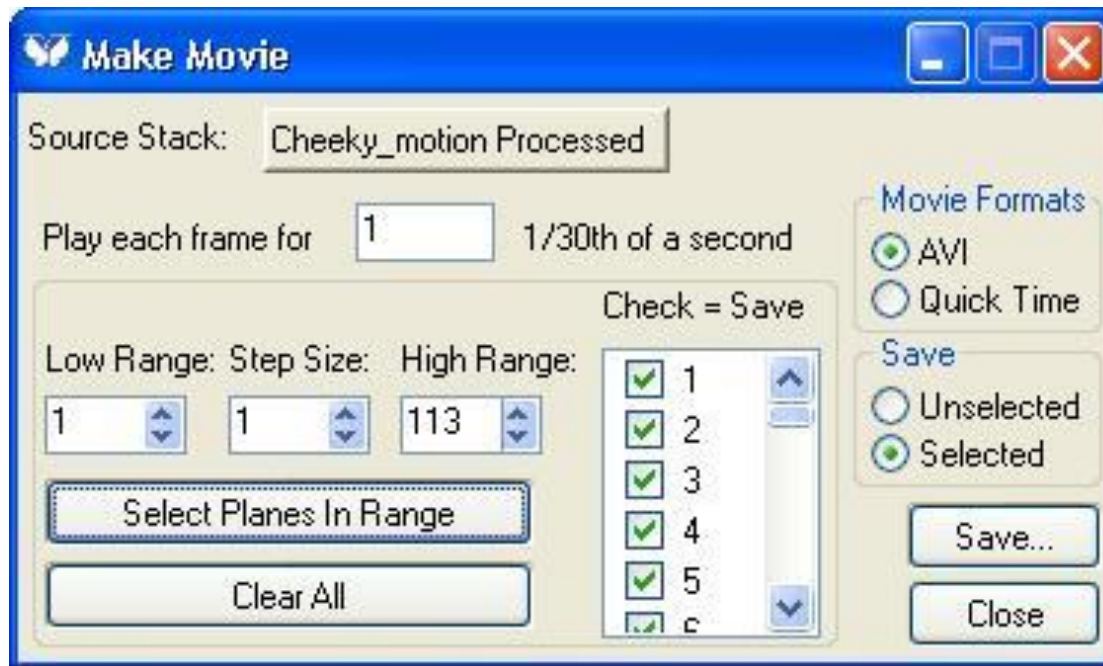


- What are Stacks?
 - 單一檔案包含多張影像

- Time Series
 - Z Series
 - Wavelength Series

Make Movie

- Stack → Make Movie
- AVI or Quick Time movie
- 可自訂影片播放時間



Working with Stacks

➤ Stack → Montage

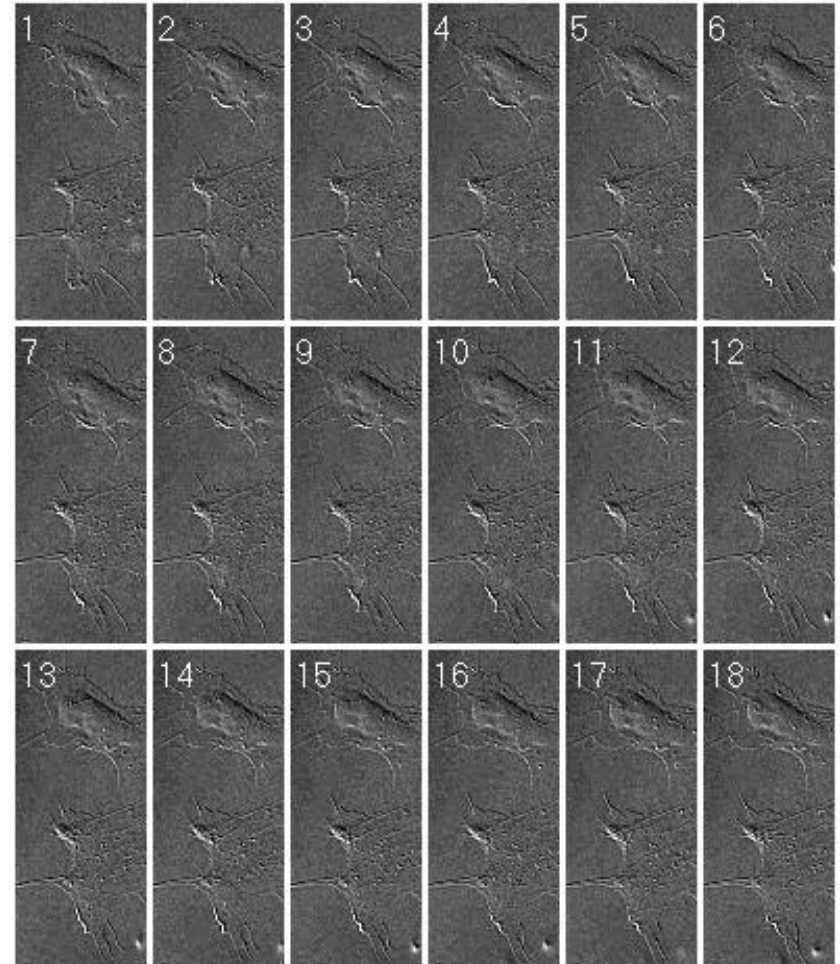
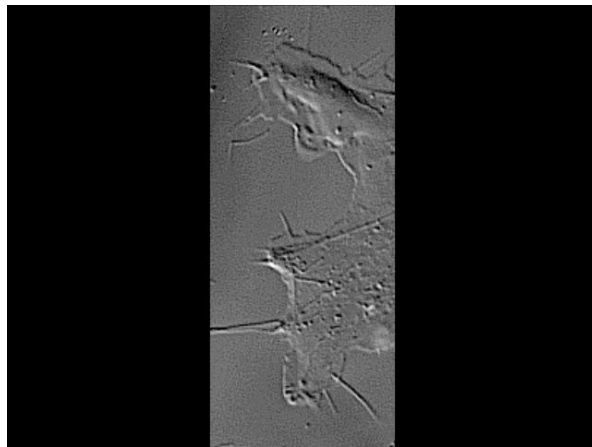
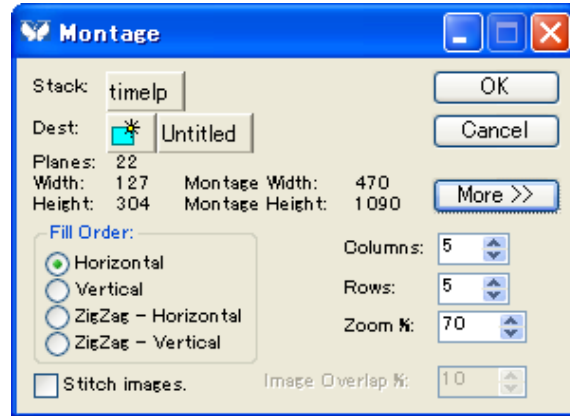
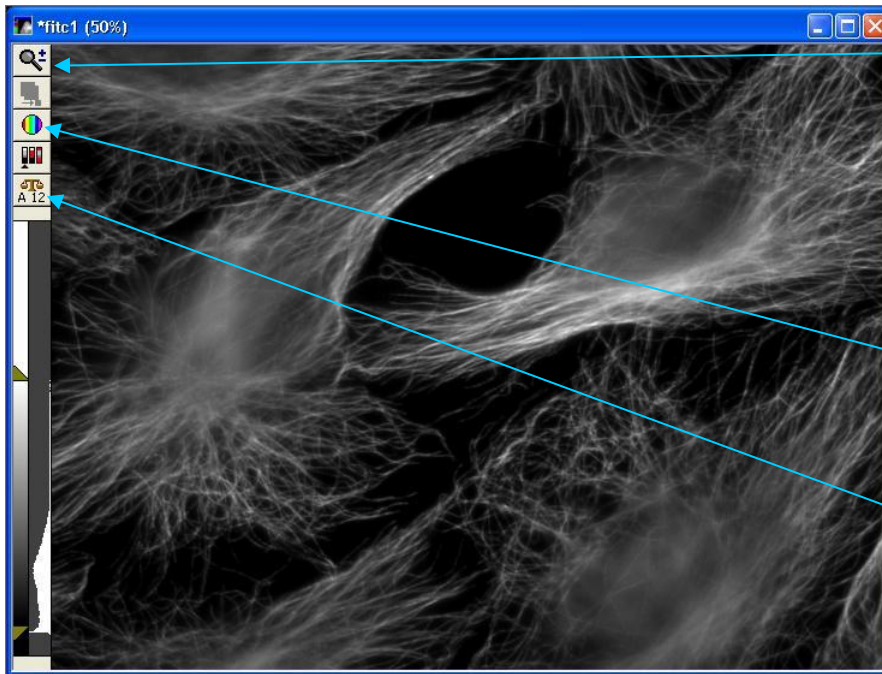


Image Window Tools



- Zoom ±
調整影像視窗放大或縮小,
也可利用滑鼠的滾輪調整
- Look Up Table(LUT)擬色
可將單色的影像做不同顏色套色
- Scale Image

Image Information



➤ Edit → Image Info or Alt-I or



The screenshot shows the 'Image Info' dialog box in the MetaMorph software. The main window displays a grayscale image of a cell. The 'Image Info' dialog is open, showing a table of properties for the image 'fitc_bin2'. A red arrow points from the 'Show Annotation >>' button to an expanded 'Annotation' window.

Property Name	Property Value
Location on Disk	C:\MM\images\fitc_bin2.stk (Plane 21)
File Type	Metamorph Stack File Format
Creation Timestamp	Tue Feb 19 15:48:45:423 2002
Last Saved Timestamp	Thu Apr 4 16:44:24:684 2002
Lookup Table Model	Monochrome
Storage Requirement(Megabytes)	2.06 MB
Image Width	150
Image Height	150
Image Depth (bits)	16
Image X Calibration (pixel/pixel)	2
Image Y Calibration (pixel/pixel)	2
Number of Planes	48
Plane Group Label	

Plane Number: 21

Show Annotation >>

Open Log Configure Log... Image Status Bar... Print... Close

Data Log Not Open

Annotation:

Exposure: 100 ms
Binning: 1 x 1
Region: 526 x 488, offset at (634, 338)
Acquired from MV-1 500
Subtract: Off
Shading: Off
Gain: 1
Light Mode: Low
Offset: 1

Copy of C03-FITC + 240
Duplicated from Fitc at 50%

Image Bit Depths



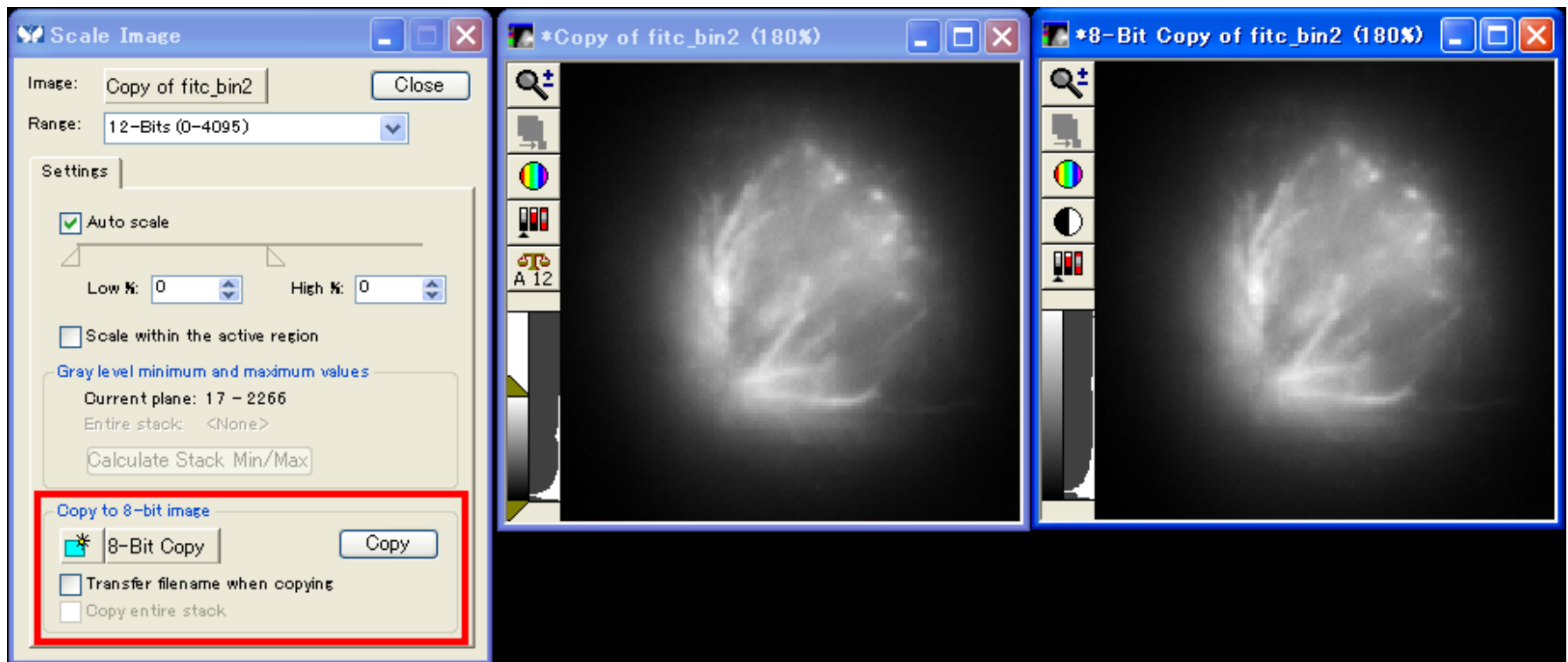
- Bit depth defines the number of values available to each **pixel** within an image
- Function of the camera electronics and software

Bit depth	Number of gray levels
1 – bit	Black and white (on or off) “Binary”
8 – bits	256 gray scale
12 – bits	4,096 gray scale
16 – bits	65,536 gray scale
24 – bits	Color image 8bits Red, 8 bits Green, 8 bits Blue

Presentation Image



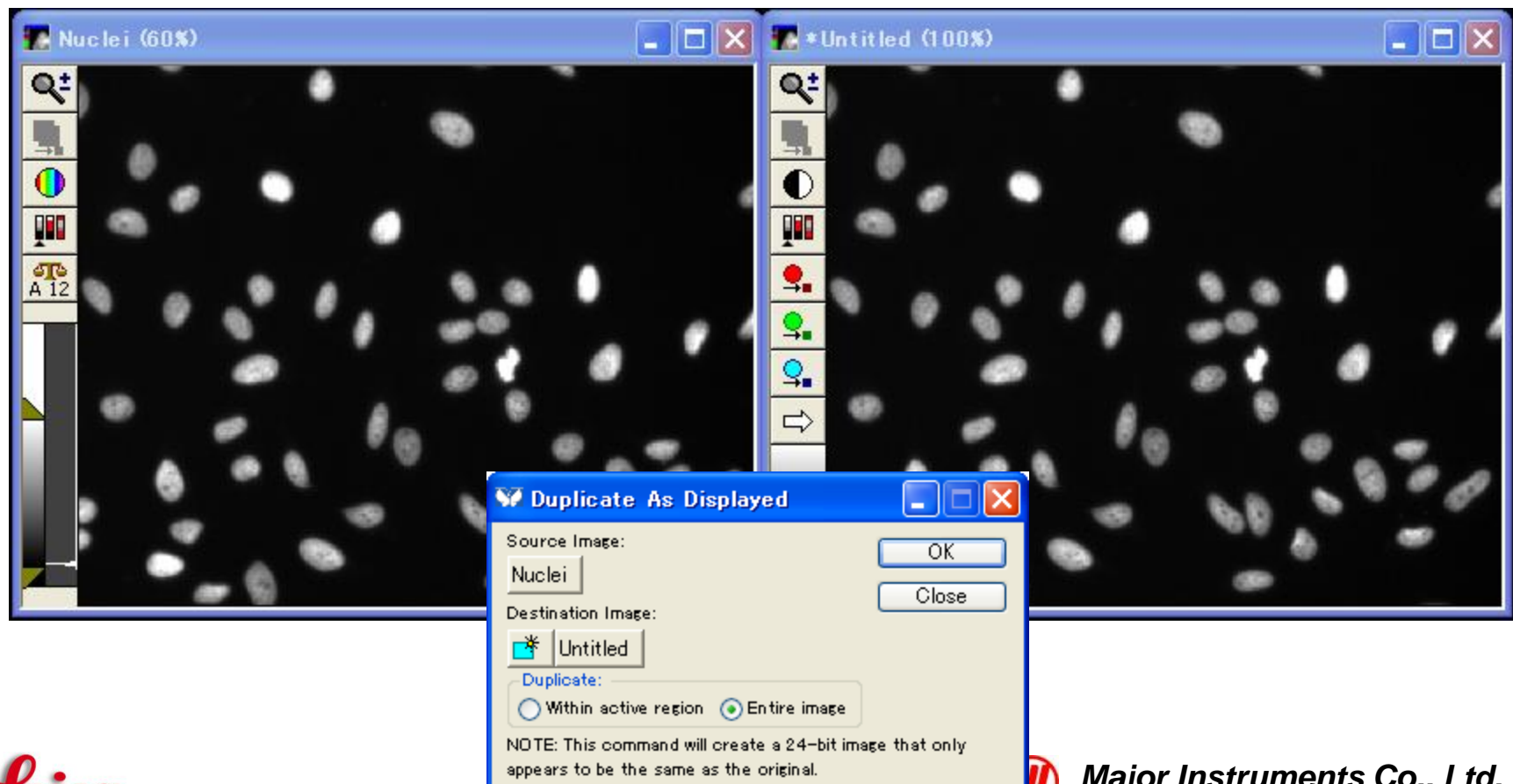
- 8 Bit Copy: Create an 8 bit copy
- 影像的原始資訊會被改變！



Presentation Image



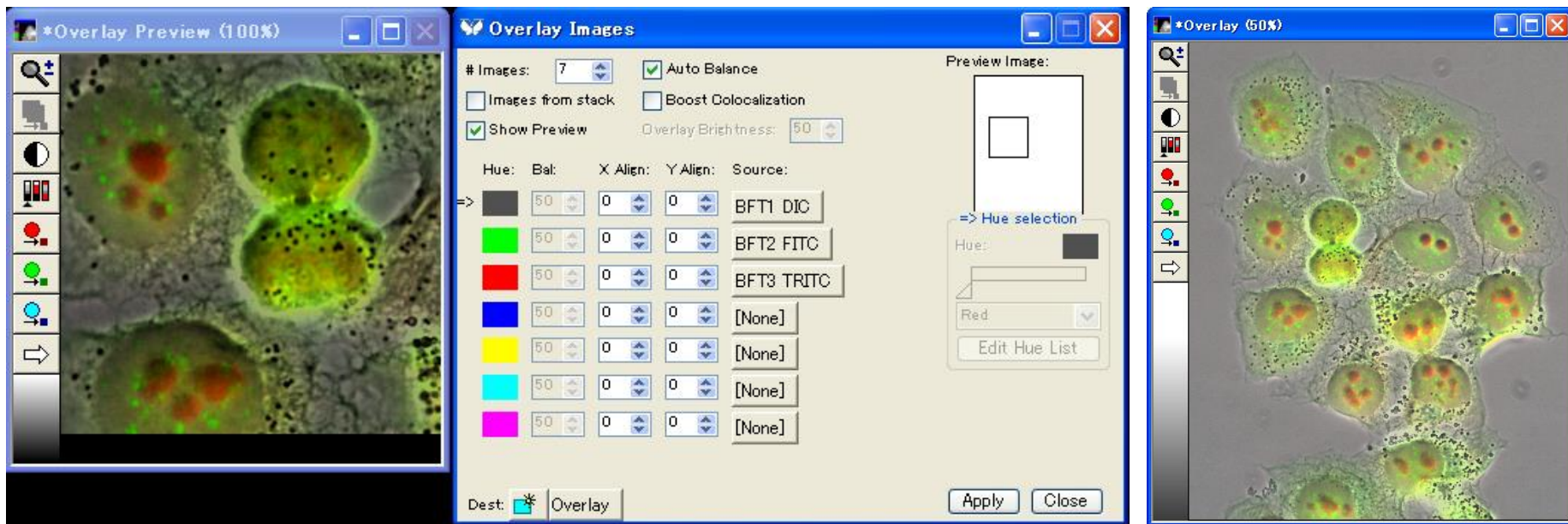
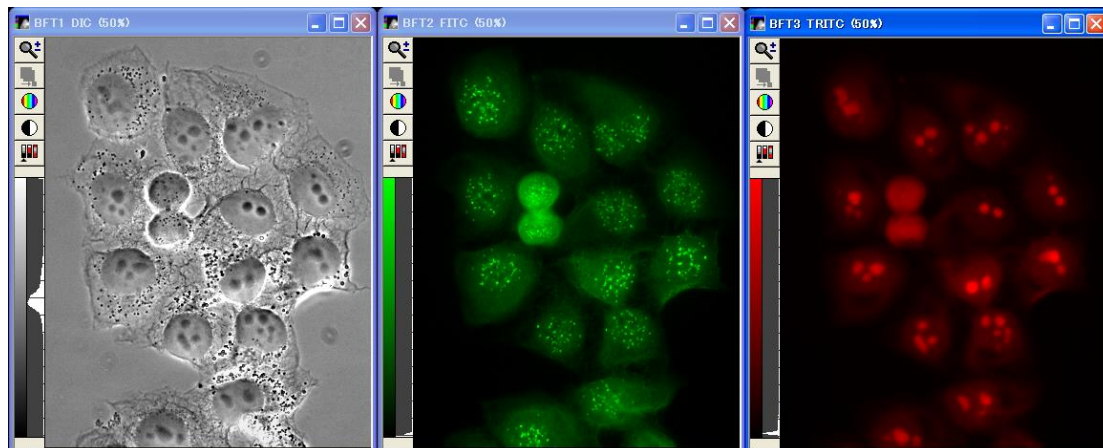
- **24 Bit Copy: Edit menu → Duplicate As Displayed**
LUT, measurement overlays, scaling...
- 影像原始資訊會被改變！



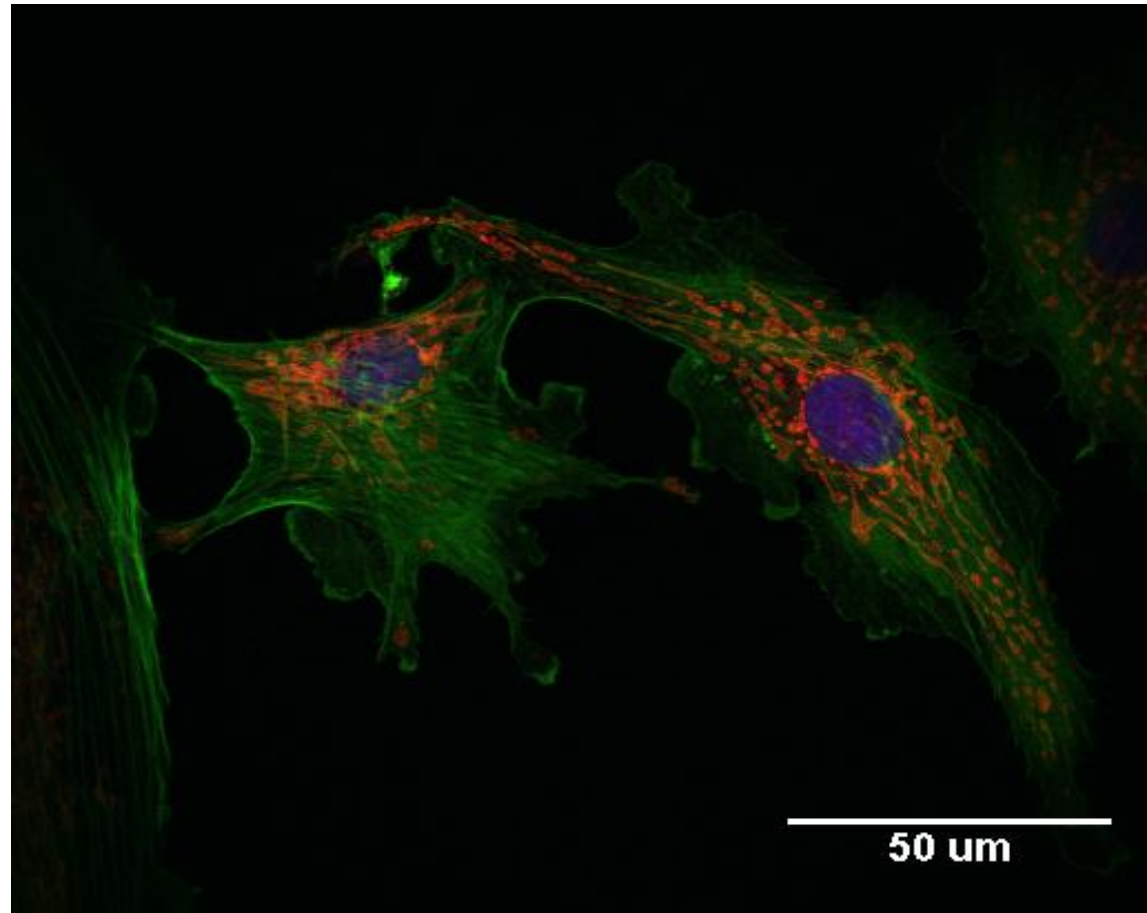
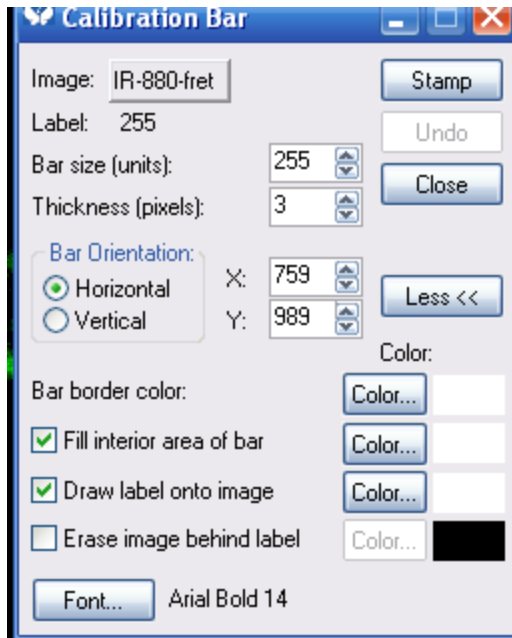
Color Overlays (Overlay Image)



- Display → Overlay Images
- 最多可重疊7個不同顏色
- 可預覽
- 可作XY align



➤ Display → Graphics → Calibration Bar



MetaMorph® imaging system

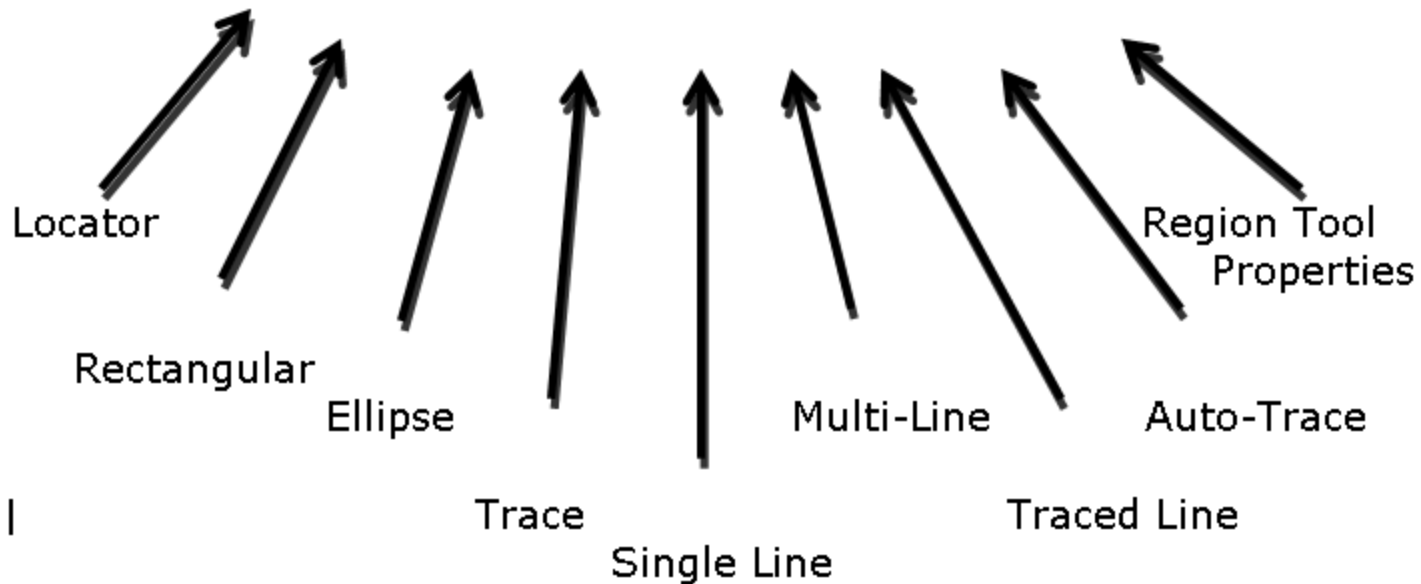
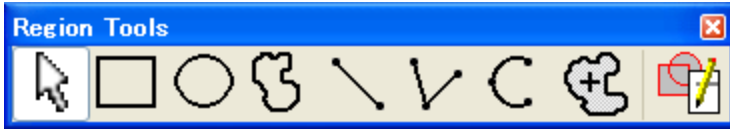


Basic Measurements

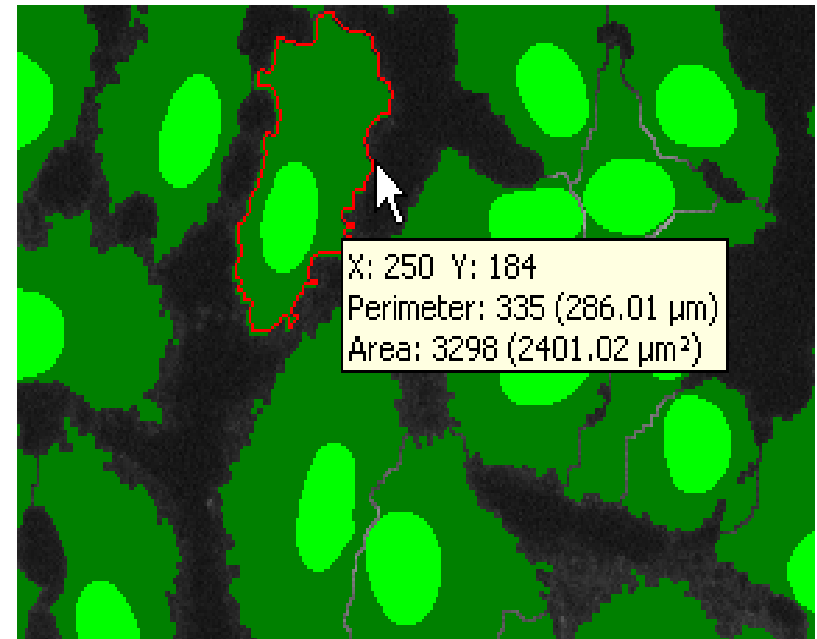
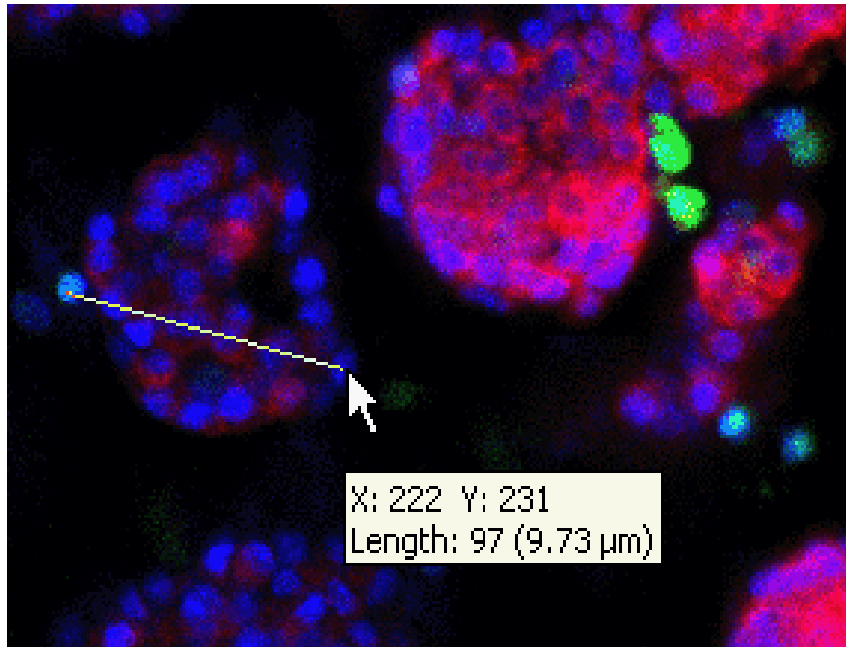


- Calibrate Distances
- Region Measurements
- Linescan
- Integrated Morphometry Analysis

Measure region -- Region of Interest (ROI)



Region Tooltips Info



長度和面積以校正後之單位顯示

Region Measurements



- Measure → Region Measurements
- 可同時量測多個圈選區域
- 對於單張影像或是多個影像堆疊檔都可進行量測
- 量測數據顯示在介面上或是可用圖表顯示

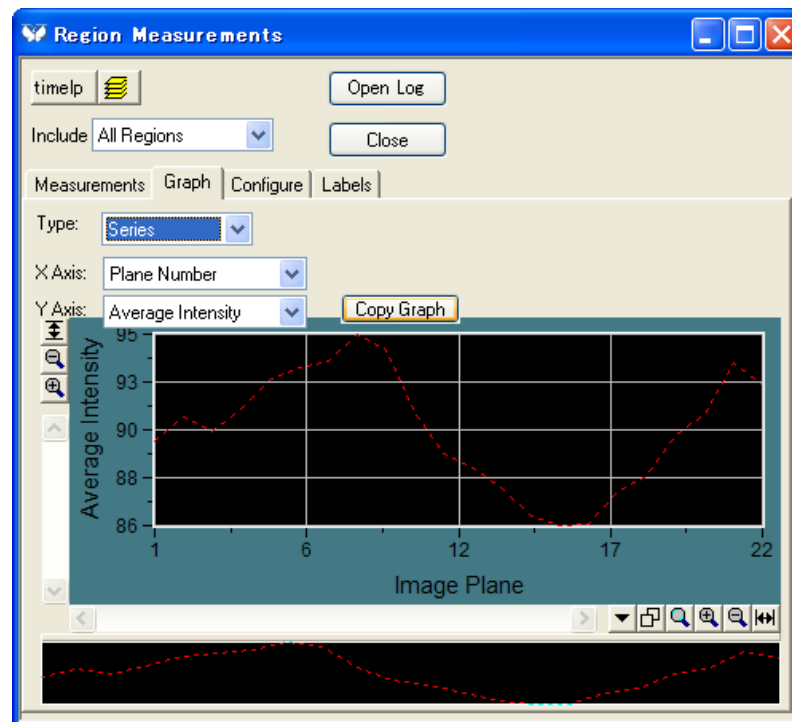
Region Measurements

timelp [icon] Open Log

Include: All Regions [dropdown] Close

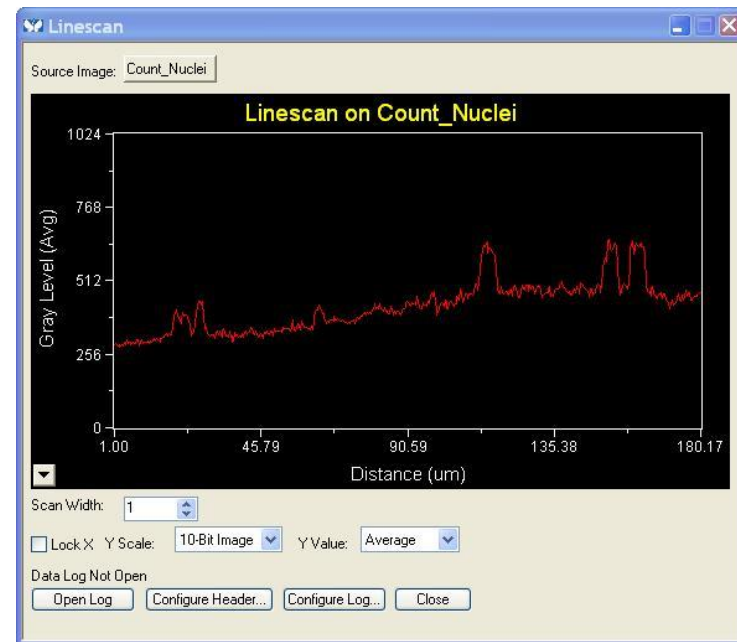
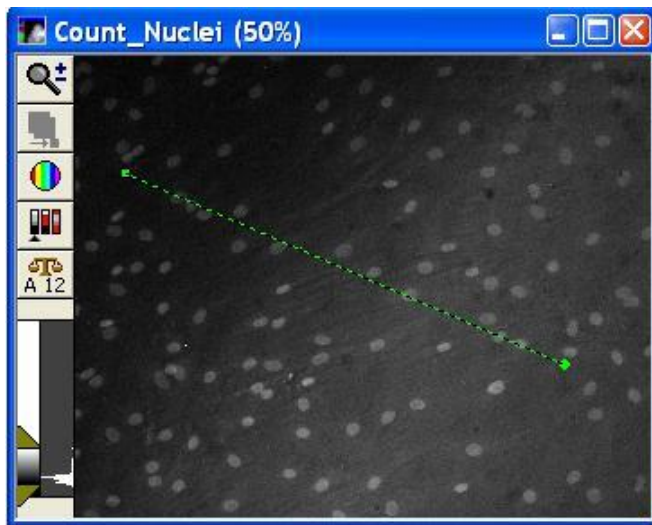
Measurements | Graph | Configure | Labels

Region Label	Image Plane	Average Intensity	Minimum Intensity	Maximum Intensity
1	1	89.8197	8	255
1	2	91.082	12	186
1	3	90.377	10	173
1	4	91.4918	0	185
1	5	93.0656	2	191
1	6	93.6066	4	187
1	7	93.9344	5	185
1	8	95.3115	2	201
1	9	94.5246	8	177
1	10	91.3443	29	145
1	11	89.1803	57	147
1	12	88.4918	59	148
Integrated	-	1989.93	752	3648

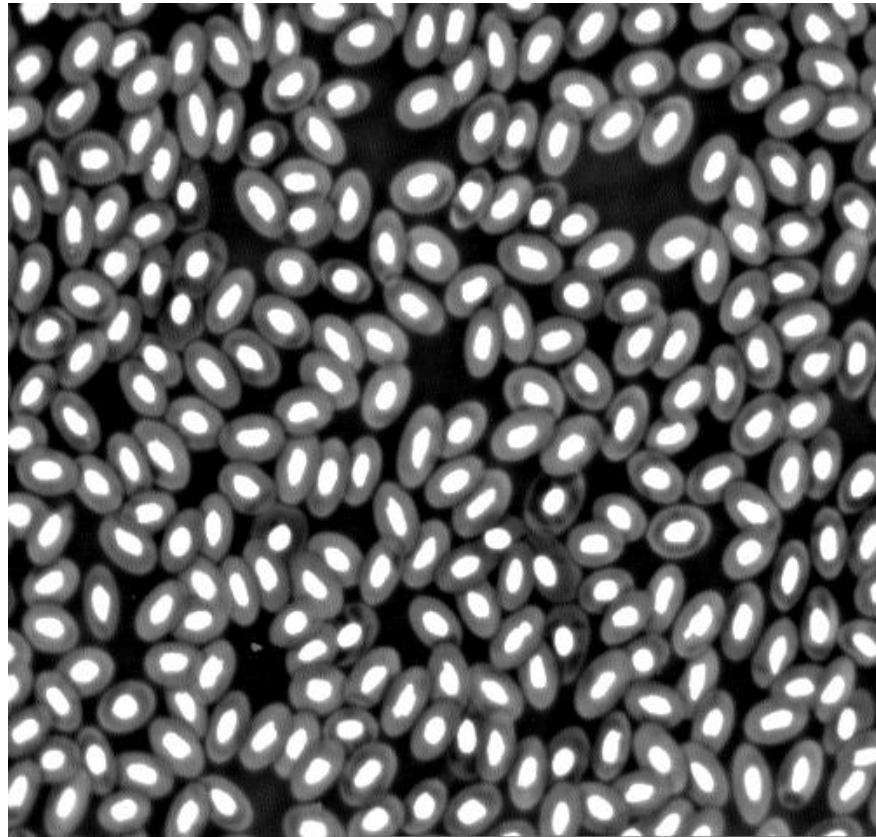


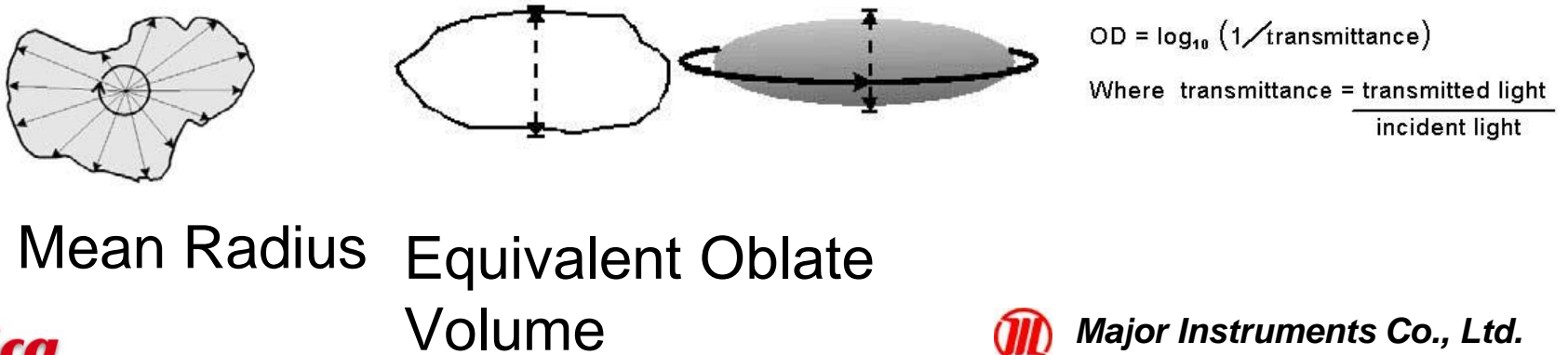
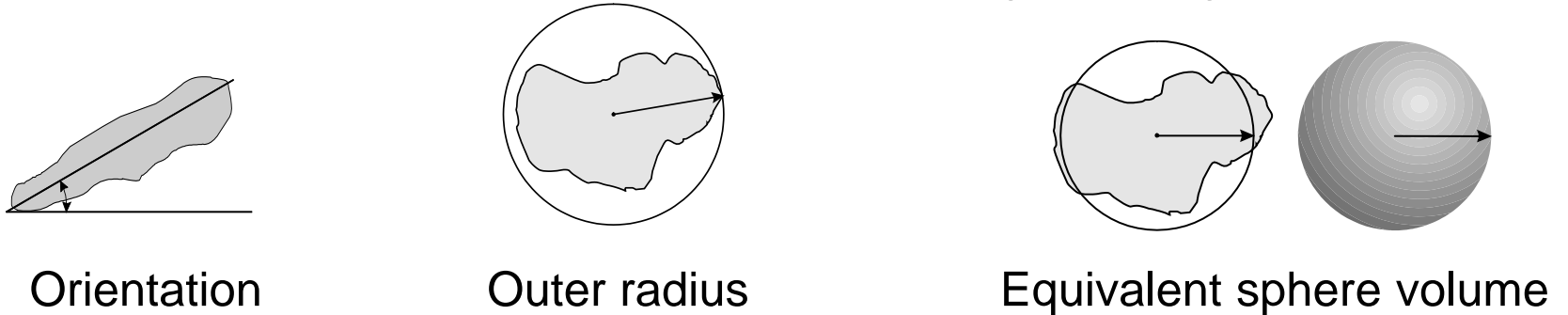
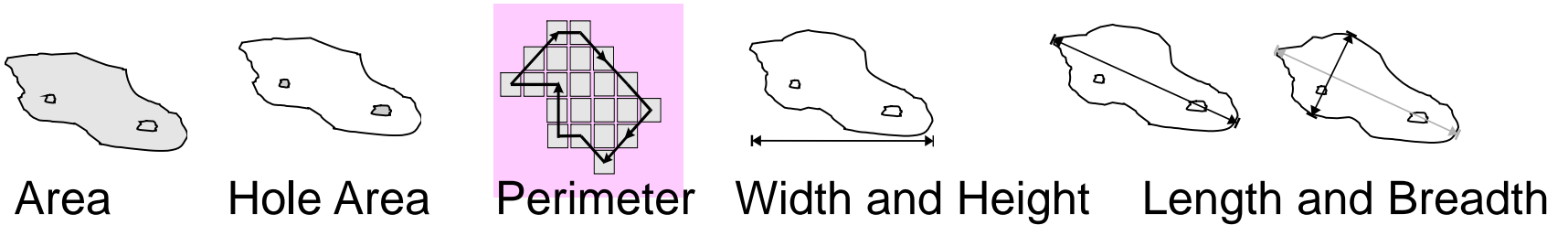
Line Scan

- Measure → Line Scan
- 拖曳的線條可顯示經過的強度分布
- 可方便使用於區分背景與物體的強度差異
- 一次只能畫一條線



Integrated Morphology Analysis

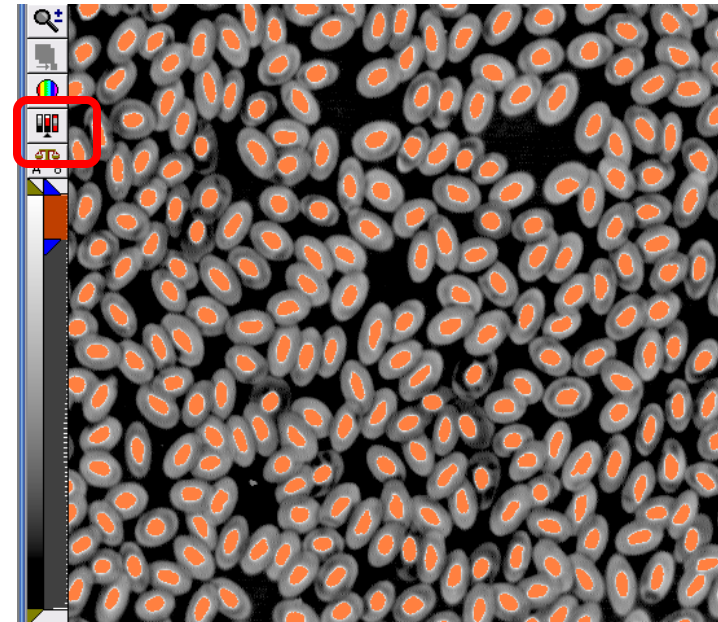
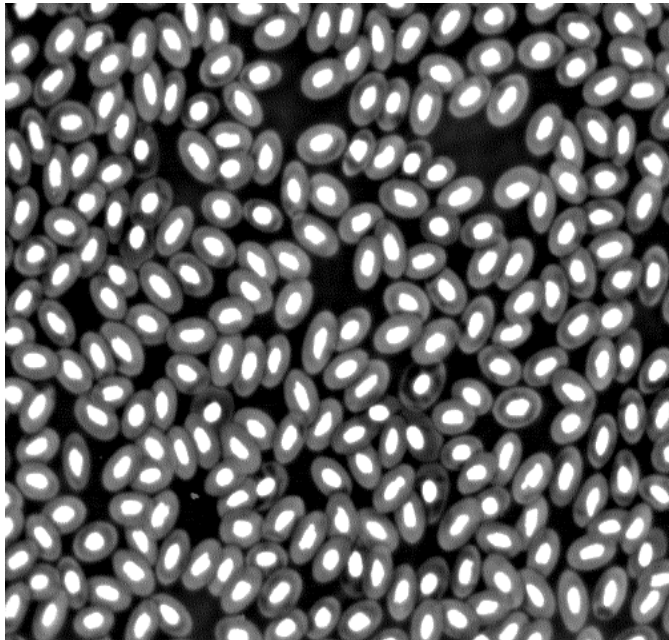




Thresholding



- Measure → Threshold Image
- 以Intensity 篩選欲觀測的區域



Integrated Morphometry Analysis



The screenshot displays the MetaMorph software interface. On the left, a window titled '*frogegs (100%)' shows a grayscale image of frog eggs with green outlines. On the right, the 'Integrated Morphometry Analysis' window is open, showing a table of measurements for 14 objects.

Source: frogegs
 Segment using mask Mask: [None]

Measurements | Preferences | View | **Object data** | Summary | Histogram | ScatterPlot

Display mode
 Current Accumulated
Object Log Not Open

Open Log | Configure Log... | View Log...

Object #	Total area	Average intensity	Intensity center X	Intensity center Y	Perimeter	Centroid X	Centroid Y
1	111.000	1.919	13.704	5.549	41.556	14.910	3.568
2	62.000	1.694	70.286	3.429	29.899	69.677	2.355
3	151.000	1.570	141.367	10.595	51.213	139.636	9.901
4	126.000	1.762	183.473	5.284	44.142	183.524	3.484
5	47.000	1.723	214.741	3.185	25.899	216.383	2.574
6	30.000	0.600	297.500	0.333	23.657	293.500	0.867
7	50.000	3.120	403.577	3.250	29.071	404.100	1.620
8	79.000	1.671	477.977	4.932	32.142	479.823	3.228
9	134.000	0.985	256.068	10.909	46.142	256.254	12.776
10	132.000	0.523	367.522	8.043	40.385	366.924	9.682
11	152.000	1.717	334.310	13.632	48.042	334.184	13.658
12	126.000	0.952	239.675	13.900	44.142	238.976	15.944
13	135.000	2.400	465.685	15.028	42.870	464.037	14.170
14	130.000	2.031	157.307	18.602	43.213	158.623	19.715

Measure | Reset Current | Load State...
Create Object Mask | Reset Accumulated | Save State... | Close

Integrated Morphometry Analysis



The screenshot displays the MetaMorph software interface. The main window shows a grayscale image of frog eggs with green outlines. The 'Integrated Morphometry Analysis' window is open, showing the 'Summary' tab. The 'Source' is 'frogeggs' and the 'Mask' is '[None]'. The 'Display mode' is set to 'Current'. The 'Summary Log Not Open' message is visible. The table below shows the following data:

Summary	Area	Average	Total	Shape factor	Width	Heig
Count	265	265	265	265	265	
Average	127.679	1.624	203.004	0.875	12.660	14
Std. Dev.	25.845	0.591	74.256	0.084	2.762	3
Minimum	4.000	0.028	3.000	0.566	1.000	2
Maximum	169.000	5.250	402.000	1.000	19.000	23
Total	33835.000	430.283	53796.000	231.822	3355.000	3879

Buttons at the bottom include: Measure, Reset Current, Load State..., Create Object Mask, Reset Accumulated, Save State..., and Close.

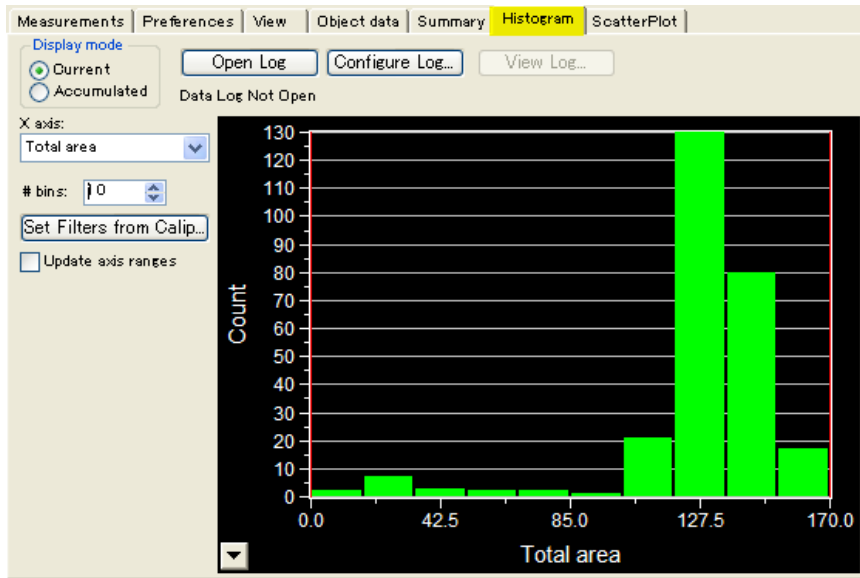
Integrated Morphometry Analysis



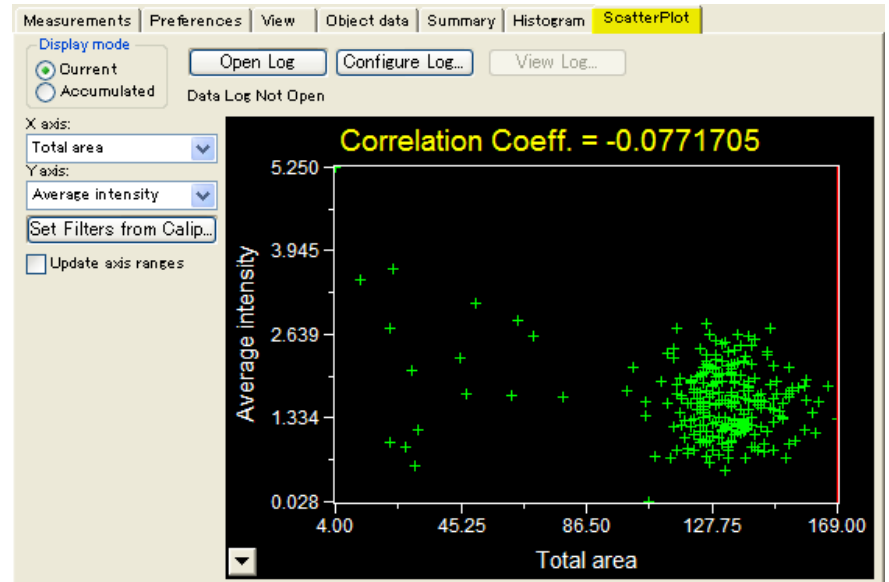
The screenshot displays the MetaMorph software interface. On the left, a microscopy image shows several green segmented particles on a black background, with one yellow particle. The window title is '*05032006 Plate 1 - 40X Compounds plus NGF_A03_s5_w1 (30%)'. On the right, the 'Integrated Morphometry Analysis' window is open, showing a table of measurements for 10 objects. The table columns are Object #, Area, Average, Total, Centroid X, and Centroid Y. Object 6 is highlighted in blue.

Object #	Area	Average	Total	Centroid X	Centroid Y
1	71.010	2576.735	7037064.000	75.635	4.835
2	166.618	2270.446	4549018.000	68.192	21.413
3	76.939	2422.656	7168638.000	87.167	24.547
4	102.342	2737.129	0773339.000	25.052	47.224
5	93.892	2706.412	9772855.000	51.065	48.473
6	245.143	3230.788	0459872.000	166.684	53.523
7	109.441	2639.249	1108598.000	104.118	53.253
8	143.269	2750.028	5152653.000	120.904	77.241
9	76.081	1672.079	4892504.000	36.536	88.565
10	92.696	2313.249	8246734.000	164.637	97.114

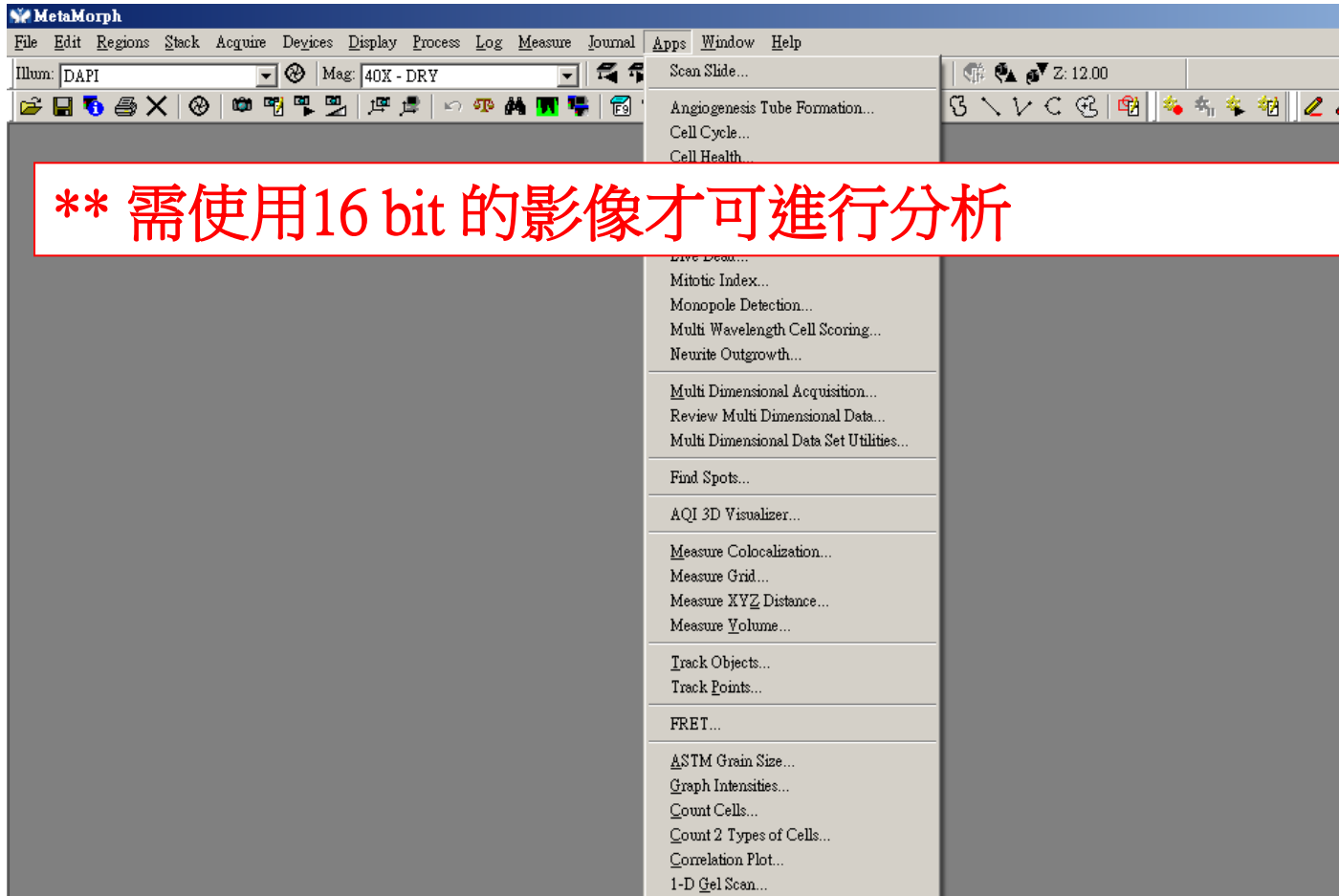
Histogram



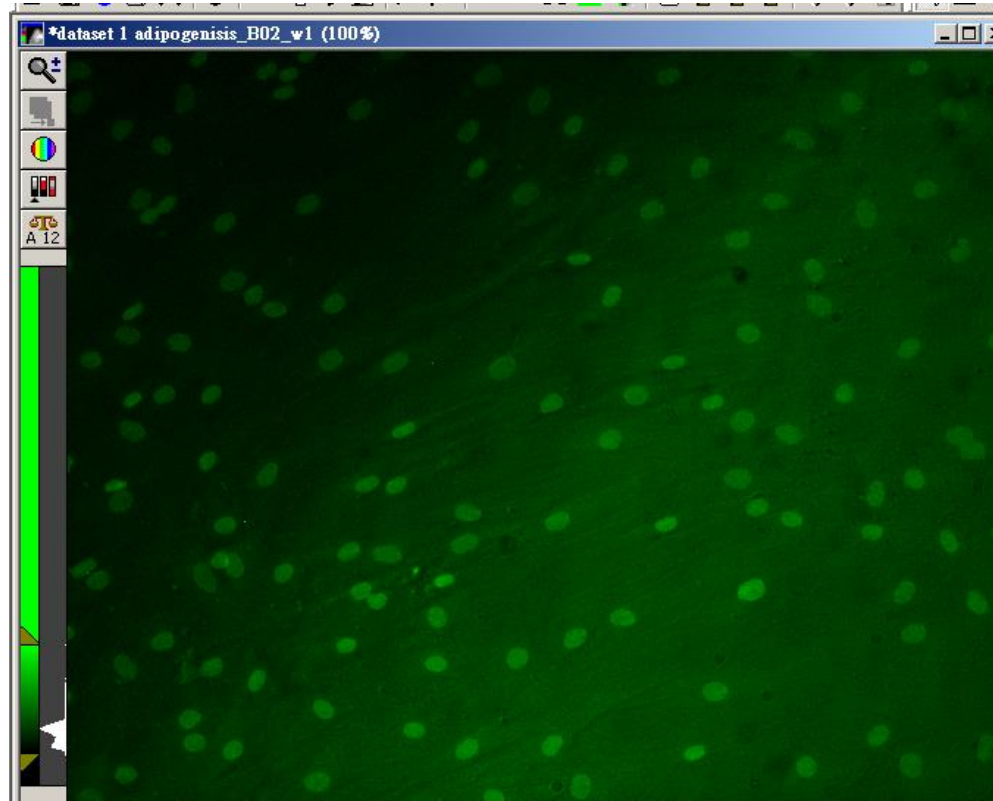
ScatterPlot

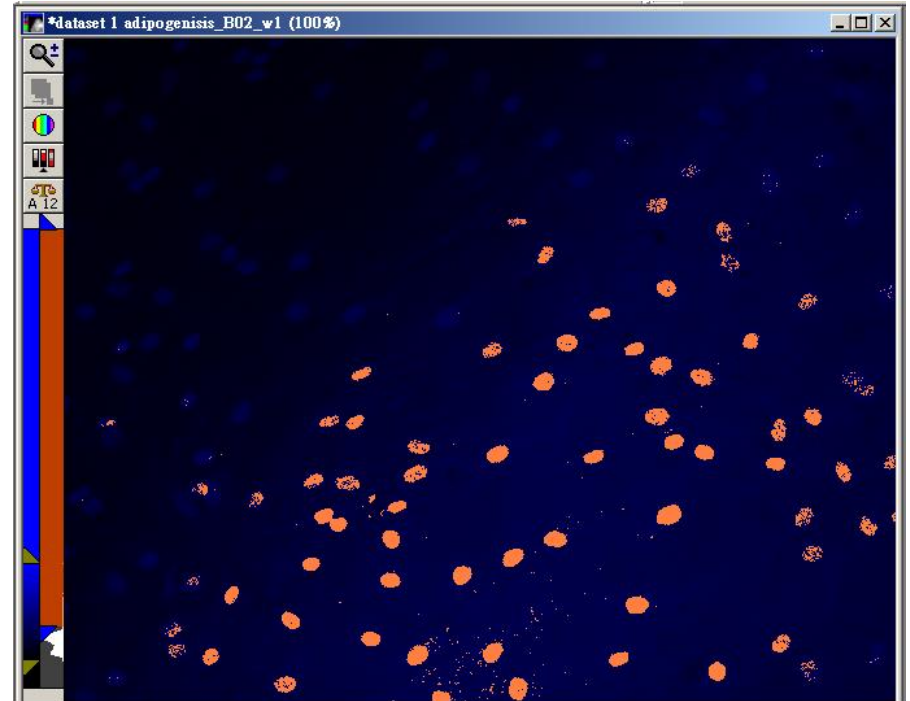
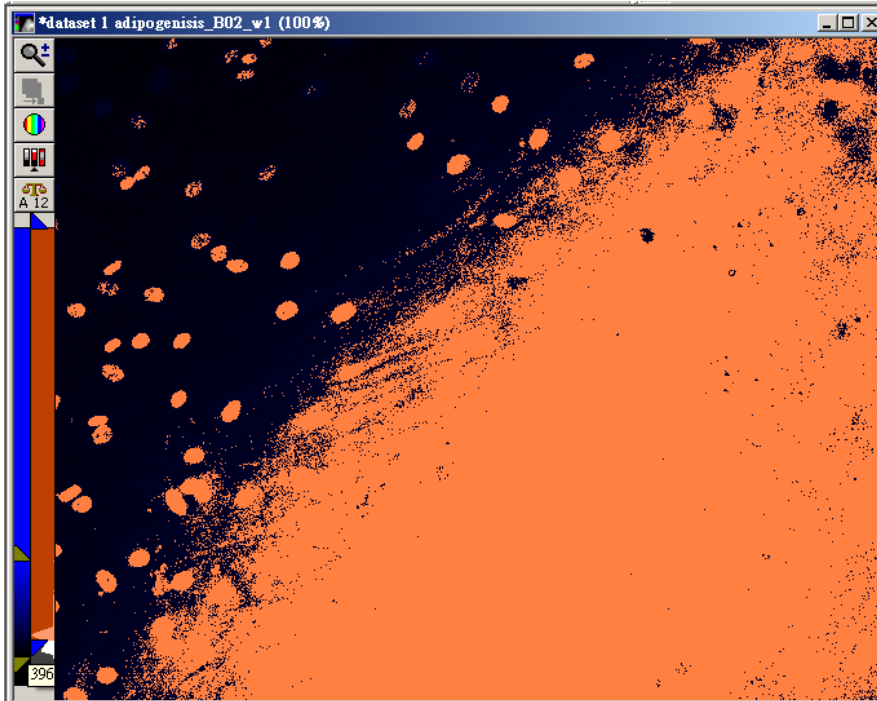


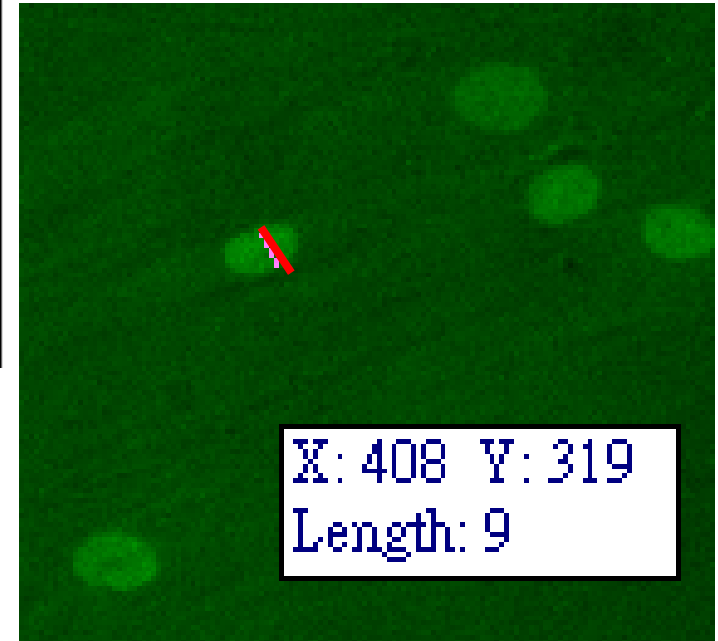
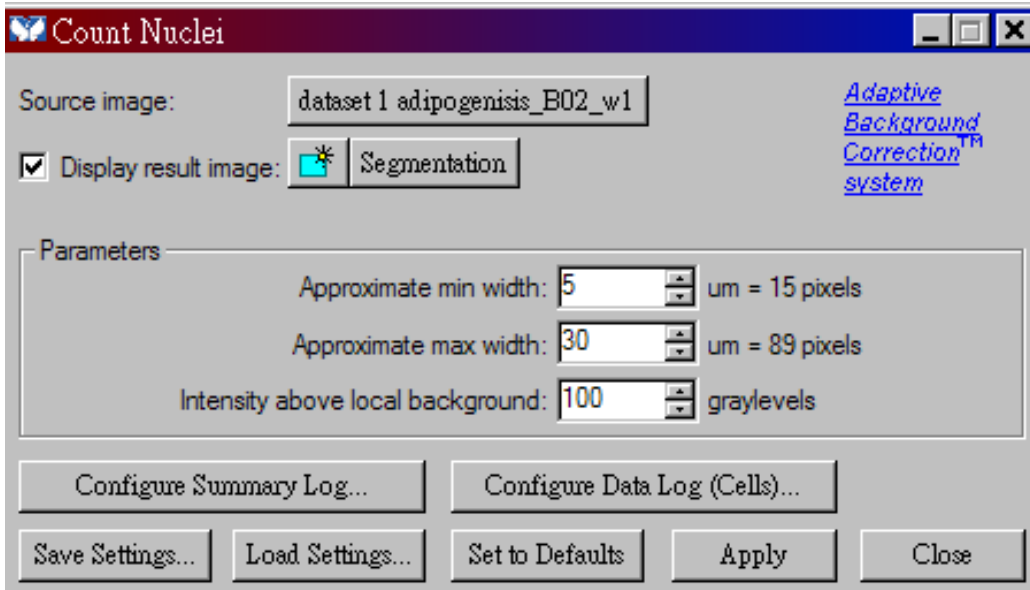
Application modules



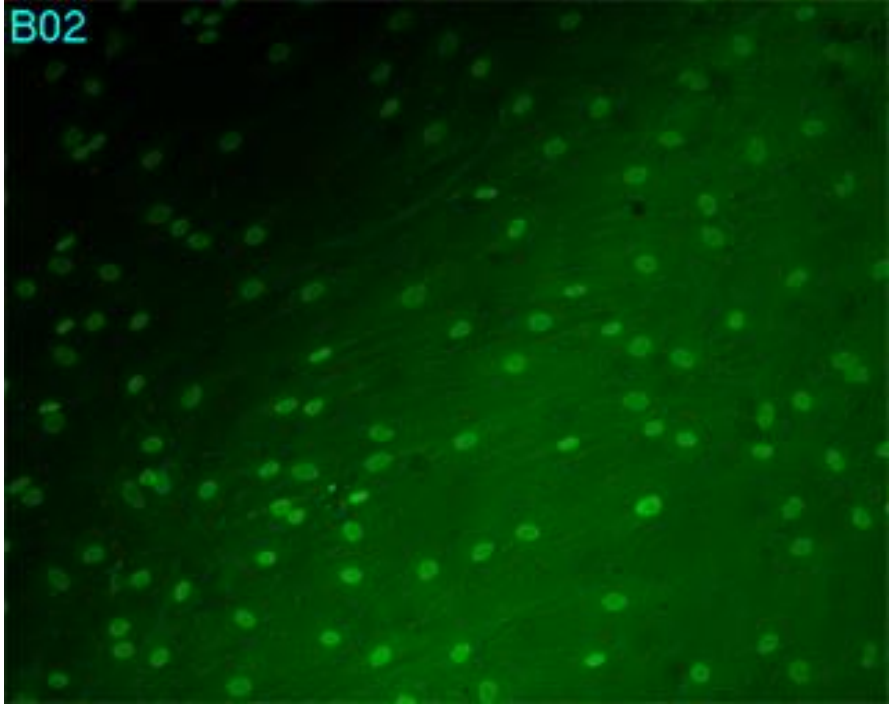
Count Nuclei





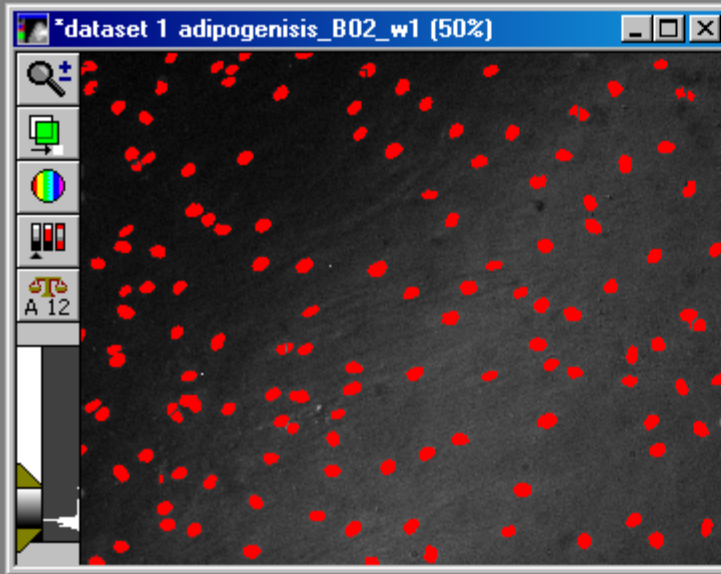


B02



etaMorph Offline

Edit Regions Stack Display Process Log Measure Journal Apps Window Help



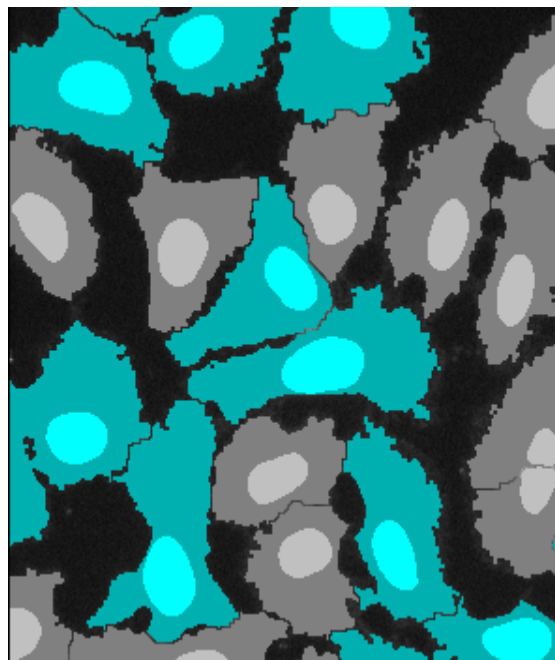
Cellular Results for Count Nuclei

	Cell: Assigned Label #	Cell: Area	Cell: Integrated Intensity	Cell: Average Intensity
1	1	3.30917	10211	352.103
2	2	3.65149	13868	433.375
3	3	3.7656	14929	452.394
4	4	3.87971	12689	373.206
5	5	4.33615	17070	449.211
6	6	6.50422	31008	544
7	7	7.07477	28911	466.306
8	8	8.10175	27687	389.958
9	9	8.44408	35274	476.676
10	10	8.90052	35889	460.115
11	11	9.24284	28585	352.901
12	12	9.24284	33952	419.16
13	13	9.69928	37977	446.788
14	14	10.1557	34049	382.573
15	15	10.3839	48343	531.242
16	16	10.6122	41748	448.903

Show Cellular Results
 Data Log Not Open

Multi Wavelength Cell Scoring Application Module

- 用途
 - *Your* research— 不需要寫程式或其他巨集就可設計多樣化的模組
 - 可使用1到7種不同的波長
 - 分析結果可針對每一個細胞及每一個波長
- 特性
 - 每一波長都可獨立進行預視
 - 針對不同研究設定波長及名稱
 - 用互動式的圖形來連結每一個獨立波長及所有波長之間的關係



Cell: Custom Profile Name	Cell: Total Area	Cell: W1 Stained Area	Cell: W2 Stained Area	Cell: W3 Stained Area	Cell: W4 Stained Area
Negative	1685.37	180.549	1685.37		
Transfected	3627.01	426.621	3627.01		
Transfected	2920.82	433.173	2920.82		
Transfected	4011.4	769.519	4011.4		
Transfected	2992.9	208.942	2992.9		
Transfected	3627.73	535.096	3627.73		
Transfected	3418.06	540.92	3418.06		
Negative	2323.85	546.016	2323.85		
Negative	1708.67	475.398	1708.67		
Transfected	3033.67	584.602	3033.67		
Negative	1887.03	394.588	1887.03		
Negative	1815.69	422.253	1815.69		
Negative	2476.73	421.525	2476.73		377.003
Negative	2363.89	428.077	2363.89		389.5

Legend [min] [max] [close]

Wavelengths:

- W1 All nuclei
- W2 Positive
- W3 Dead
- W4 Compound X

Profiles: Custom Profile Names:

- 1--- Negative
- 12-- Transfected
- 1-3- Dead
- 123- Transfected Dead
- 1--4 X Live
- 12-4 X Transfected
- 1-34 X Dead
- 1234



Thank You



Major Instruments Co., Ltd.