**NIKON PCM 2000 Confocal Microscope**

This system has been reengineered by Don Elsmore (ph: 443 745 2087). It has 2 objective lenses (40x oil & 60x oil) and has 5 acousto-optical tunable filters (AOTF), although it has the capacity for another 3. These filters allow for precise control of fluorescence excitation wavelengths from the system’s 3 lasers:

* Argon gas laser which can generate
	+ 457/8nm Excites CFP
	+ 488nm Excites GFP / FITC
	+ 514nm Excites YFP
* Solid state x 2
	+ 405nm Excites DAPI / Hoechst
	+ 552nm Excites RFP

Paperwork

* Book in advance to be sure of scope use
* Log in on folder & record time in

Hardware start up

* Ensure objective lenses are lowered with large dial on left of scope
* Turn on **1** (power to Argon laser – on shelf), then **2** (key on same box), then **3** (key on laser power level controller – box with dial on right of scope), then **4** (power switch on 552 laser box), then **5** (key on same box), then **6** (power for 405 laser - black switch on its own), then **7** (key on 405 laser), then **8** (power for AOTF - switch on side of large black box), then **9** (power to halogen lamp - box below No. 3), then **10** (remote focus control – box on left of scope), then **11** (power to photomultiplier tube (PMT) – at base of the back of black box behind computer tower), then **12** (power to computer – button at front of tower)
* Check to see that the microscope air-table if floating
* Note: Argon laser will need about 15mins to warm up

Software start up

* Double click on **Chameleon AOTF** (laser control)
	+ 2 boxes appear
		- **MDS Control** software box
			* Ensure both internal (IM mode) & 10V (V mode) are checked
		- **Laser AOTF Control** box
			* Click all light bulbs, which will turn all the lasers on
				+ Then click them all off – this will reset the system
* Double click on **Simple PCI** (imaging control)
	+ Click camera icon and the **Capture** window appears
		- Ensure **Park Off Sample** box is always checked
	+ There are 2 ports at the front of the PC tower for flash drives

Operation

* Adjust wheel on right side of scope to **A** when viewing bright field
	+ Turn to **C** when using laser
* Add small drop of oil to chosen lens (DO NOT TOUCH LENS WITH GLASS APPLICATOR)
* Raise objective lens with large focus wheel on left of scope until oil contacts slide
	+ DO NOT USE SMALL WHEEL, as it is motorized and controlled by dial marked **10**
* Use remote focus control wheel (box **10**)to bring slide into focus
	+ Box has switch on right side that gives 3 settings; top to bottom
		- Fine; medium; coarse
	+ The intensity of the halogen light can be adjusted by the knob on power source and can only be shut off by turning the power off (switch **9**)
* Focus with bright field may be difficult and so may need to be done with laser illumination
	+ Wheel with lever at front of scope brings a target into view through eyepieces
* Set required light path on Scanner Module (black box with blue levers on left side of scope)
	+ See light pathways on diagram below
	+ **Excitation Filter** doesn’t contain a filter – should always remain pushed in
	+ **ND Filters** – sealed up to prevent dust getting in
	+ Position 1 is when levers are pushed all the way; position 2 is the next up etc.
	+ **Pinhole** – adjusts the amount of laser light entering the system
		- Position 1 = 22µm diameter
		- Position 2 = open pinhole with a 25% neutral density (ND) filter (all wavelengths equally dimmed); inserted to prevent PMT damage during change of pinhole
		- Position 3 = 52µm diameter
	+ Dichroic mirrors
		- These split light into two separate beams with differing wavelengths; usually meaning that below a set value light will be reflected at 90O and above that value light will pass through (transmitted)
		- Excitation (**Dex** – dichroic excitation)
			* Reflects light from lasers that has passed through excitation filter and ND filter (none) on to sample, but allows emitted light to pass through
			* 3 settings
				+ **RGB** – reflects 405, 488, 552 & 633/5 (don’t have) on to sample

Allows transmitted light to pass through in peaks

* + - * + **505** – only reflects light below 505 on to sample

Allows emitted light above 505nm to pass through

* + - * + **457/514** – only reflects 457 & 514 lasers

Allows all transmitted light to pass through in peaks

* + - Emission
			* **Dem1** – (dichroic emission 1) has 4 settings
				+ **RF** – reflection full => all emission light goes to BA1 (blue)
				+ **495** – light below 495nm goes to BA1; rest goes on to Dem2
				+ **HM** – half mirror => half light is reflected, half is transmitted
				+ **OP** – open, no dichroic => all light is passed on to Dem2
			* **Dem2** – has 4 settings
				+ **RF** – all emission light that arrives here goes to BA2 (green)
				+ **565** – light below 565nm goes to BA2; rest goes on to BA3 (red)
				+ **HM** – half light is reflected to BA2; half goes on to BA3
				+ **OP** – all light is passed on to BA3
	+ BA filters = barrier filters
		- These are emission filters and should always be pushed in
		- **BA1** – 450nm (35nm band width) – Blue
		- **BA2** – 515nm (30nm band width) – Green
		- **BA3** – 583nm (30nm band width) – Red
		- Note: We also have another BA filter which is a 510 long pass if needed
	+ PMT – photomultiplier tubes detect photons arriving through the BA filters and then multiply the photoelectric current produced by those photons by up to 100 million times
		- **PMT1** – receives light from BA2 (green)
		- **PMT2** – receives light from BA3 (red)
		- **PMT3** – receives light from BA1 (blue)
* When ready to start imaging check wheel on right of scope is set to **A** then click **AOTF Controller**
	+ Click **light bulbs** for lasers required to excite experimental fluorophores/dyes
	+ Click **box** around light bulb (turns dark gray when selected)
		- Intensity of laser can now be adjusted on **vertical scale**
			* Start with **10dBm** (decibel milliwatt i.e. 10 x log 10 scale)
				+ For DAPI 405nm needs to be at full power
		- DO NOT alter frequency slide on horizontal scale
			* If altered by mistake
				+ Click **Device Setup**

Click **Read from MDS**

Uncheck channels 3, 4 & 8

* Return to **Simple PCI** controller
	+ **Fast Scan** starts low quality imaging for focus and image adjustments
		- To prevent bleaching adjust the **Fast Scan Mode** under **Scan Control**
			* It is set at 1x Fast scan; can increase to 2x or 4x to reduce bleaching
	+ **Black Level Offset** will all start at 350 and should not need adjusting
	+ **PMT Gains** will all start at 1024 and will need to be adjusted to optimize images
		- Gains should be increased with caution above 2000 and never be > 3000
		- If the light entering any PMT channel is intense enough to potentially damage the photomultiplier that channel will automatically shut off and red LEDs will appear at the back of the PMT tower; if this happens then:-
			* Click button to the right of the PMT channel affected
			* The top button (contains all 3 colors) will reset them all
	+ **Capture** – takes continuous images
	+ **Capture 1** – takes a single image
		- To reduce the noise in an image click the **Processing** tab
			* Then click **Noise Reduction** in the **Operation Type** menu
				+ Select **Rolling Average** under the noise reduction menu

Starts at 4; set at 8 for improved but slower image

* + To save an image click on the save icon at the top left of the **Capture** window (NOT the **Save** button)
		- Select your lab’s folder on the **Images D: drive**
			* Select the format you wish to use to save the image (usually TIF 8 bit)
* To take a **Z-stack image**
	+ Focus on image and reset remote control (red button on box 10) either at top or bottom
		- Focus through image and note new position (numbers on remote control)
	+ Ensure that data is being saved in the correct drive
	+ Click **Sequence**
		- Give the file a name and click **Save** (default file type is **.cxd** ( Simple PCI format)
			* Select **Z scan** & **Pattern**, then click **Next**
				+ Set **Start** & **End** (zero & number noted above on remote control)
				+ Set required **Increments** (in µm) then click **Next**

Select **To a Single File** & **Save to Disk** then click **Finish**

Click **Start**

* To take a **Time series image**
	+ Ensure that data is being saved to the correct drive
	+ Click **Sequence**
		- Give the file a name and click **Save**
			* Select **Time Scan** & **Pattern**, then click **Next**
				+ Set **Start** & **End**
				+ Set required **Increments** (in seconds) then click **Next**

Select **To a Single File** & **Save to Disk** then click **Finish**

Click **Start**

* To view saved images
	+ Must close down the **Capture** window
		- On main **Simple PCI** window click file open icon
			* Multiple tools are available for image analysis once open
				+ Once altered the new image can be saved in the normal manner
* When changing slides the lens and slides should be cleaned with original Windex and lens paper to fully clear off any oil

Shutting down

* Clean and then lower the objective lenses
* Turn on and then off all lasers with **AOTF Controller** and then turn off switches in reverse order
* After turning off key **2** wait for 10-15mins before switching off button **1** to cool off laser
* Place mouse back on charging stand

Troubleshooting

* Difficulty viewing image in bright field
	+ Check wheel on right of scope is set to **A**
	+ Check **polarizer** on right side of objectives is in “out” position
* Difficulty viewing confocal image
	+ Check laser appearing in objective lens (DO NOT LOOK STRAIGHT INTO LENS), if not
		- Check wheel on right of scope is set to **C**
	+ Check laser spill in AOTF (large black box), if present
		- Turn on lasers on and off with **AOTF Controller**
		- Click reset button next to button **8**

