DEPAR FFPE TISSUE MEASUREMENT PROTOCOL FOR ROUND-ROBIN EXPERIMENT VERSION 5.0 SUMMARY

Created: 06/06/16

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A. Initial imaging, selection of areas and annotating regions of Interest from H&E sections (performed by CENTRE 1)

Macro and HiRes montage images of H&E slides

a. Open lid on the 802 by pushing 'Locked' button



b. Open Wire, click on the 'pathology workflow' button



Make sure sample illumination is to 'transmitted' and macro X0.4 is selected on the magnification panel



c. Click on the 'microscope slide 75 x 25 x 1 mm' tab and click on 'Go to Load position' button on the panel

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below:	
Not set	Microscope slide 75 x 25 x 1 mm	
Image (macro)	◎ Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	─ Tissue Holder	
Y1 = Not used		
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used		
Max = Not used	Automatically generate an image of the whole sample area	
lmage (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back	

The stage will move down and to loading position



d. Mount H&E slide into glass slide holder and load tray







e. Click 'Go to default position' to set stage position and height to default position for microscope glass slides.

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below:	
Not set	Microscope slide 75 x 25 x 1 mm	
Image (macro)	Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	Tissue Holder	
Y1 = Not used		
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used	C Automatically, and an invest of the sub-shares of the sub-shares	
Max = Not used	Automatically generate an image of the whole sample area	
lmage (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back	



- f. When the sections of interest are in view on the live video, switch to HiRes x25 and focus on one of the sections of interest. Once a section is in focus, switch back to macro 0.4x and centre the field of view on the live video using trackball or clicking on the live video window
- g. Create single snap image of the low res image using: Live video -> Snap -> Single
- h. Save the live macro image as ID_Path_Instit_HE_macro.png using Snipping tool OR Save the image by right clicking over it: Save to -> With Axes
- Export the surface file using: Export -> Surface file -> save as ID_Path_Instit_HE_macro.srf)



j. Click Next

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below:	
Not set	Microscope slide 75 x 25 x 1 mm	
Image (macro)	Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	Tissue Holder	
Y1 = Not used	- · · · · · · · · · · · · · · · · · · ·	
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used		
Max = Not used	Automatically generate an image of the whole sample area	
lmage (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back Next >	

k. Select region on the live video image of the first section to image and click next



I. Deselect 'define surface' and 'collect Raman map' and click next

Pathology 1.0 (Alpha)		
Summary	Options	
Sample Microscope slide 75 x 25 x 1 mm Image (macro)	Define surface over selected region Collect image montage over selected region I Let me review the montage before continuing	
Area of interest X1 = 20,871.8 Y1 = -6,884.4 X2 = 25.081.7	Collect Raman map over selected region	
Y2 = -4,835.6 Surface		
Min = Not used Max = Not used		
Image (hi-mag) Notset	Restart Cancel < Back Next >	

 m. The workflow will prompt the user to verify focus of section at x25 magnification (you might have to switch between macro and x25 to get representative area). Click next



n. The workflow will begin creating the montage image of the selected section



 Once the montage has been created, Screenshot and save the image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_HE_section.srf)



p. Using the x25 in live viewer you can annotate this representative hiRes image with areas of interest for Raman mapping



(Performed using 802)



(these representative images and annotations were performed using CENTRE 1's external digital microscopes and 10x and 20x objectives: top is IM and bottom is AC)

- Repeat this process for different areas of this section as well as regions of interest on other sections on this slide (and Export -> Surface file -> save as ID_Path_Instit_HE_section.srf)
- r. Eventually you will build up a collect of representative images from slide samples

15_22502



- s. CENTRE 1 will compile these representatives images, annotations and Hi-mag images into a PowerPoint document for other sites to use and reference for selecting areas of interest on the unstained sections
- t. CENTRE 1 will use these images, annotations and original H&E glass slide to get confirmation of pathology diagnosis of selected areas by first the consultant histopathologist and then blinded second opinion by other pathologists



(these representatives and annotations were performed using CENTRE 1's external digital microscopes)

B. Receipt of tissue samples and Raman Spectroscopic Measurements at CENTRE 1 and remote sites

For the remote sites, on delivery of samples, remove samples (in the slide holders) from the bubble-wrap packaging and store at room temperature for future Raman measurements. **Measure samples within a week of receipt to prevent degradation of sample quality.**

Print out digital data which has been emailed (by CENTRE 1) to the sites detailing the regions of interest selected from tissue sections on each slide.

The users at each site will select corresponding map regions at 10um pixel resolution (select 10YBin binning) resulting in ~2000 spectral points per map [not exceeding 6000 spectra points per tissue sample slide]. Each spectral point will be acquired for **6s** resulting in ~30-60 mins mapping time for each region map depending on size. See following section

CENTRE 1 will determine the order of preference for the relevant regions on a slide so that all sites are measuring the same regions from each slide. These will be annotated on the PowerPoint slides with the H&E and unstained sections. Users are asked to acquire at least 4000 spectra points in total for each section (~1000-2000 spectra per map).

C. Imaging and selection of areas of Interest from unstained sections using H&E annotated images (performed by all)

On morning of tissue measurements, perform external standard measurements using protocol: STANDARDS MEASUREMENT PROTOCOL_v2_withAppendix_190516

a. Open lid on 802 by pushing 'Locked' button



b. Open Wire, click on the 'pathology workflow' button



c. Make sure sample illumination is to 'Reflected' and macro X0.4 is selected on the magnification panel

Magnification
Sample illumination Reflected Transmitted
Macro X0.4 X0.8 X1.6 X3.2
Hi-mag X25 X50

d. Check 'automatically generate an image', click on the 'microscope slide 75 x 25 x 1 mm' tab and click on 'Go to Load position' button on the panel

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below:	
Microscope slide 75 x 25 x 1 mm	Microscope slide 75 x 25 x 1 mm	
Image (macro)	Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	Tissue Holder	
Y1 = Not used		
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used		
Max = Not used	Automatically generate an image of the whole sample area	
Image (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back Next >	

The stage will move down and to loading position



e. Take out sample slide from slide holder. Identification of the slide will be written/etched on the opposite side of the stainless steel slide (duller side) using a diamond crystal pen. The ID will also be clearly written on a white sticker on the reverse side of the slide.



f. Make sure to close slide holder to prevent dust particles from getting on the other slides in the holder

g. Carefully place the stainless steel slide into the 'microscope slide' mounting tray shiny side up making sure to orientate the slide similar to orientation of H&E section image



- **h.** Click 'Go to default position' to stage position and height to default position for microscope slides.
- i. Close lid

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below: Microscope slide 75 x 25 x 1 mm	
Microscope slide 75 x 25 x 1 mm		
Image (macro)	Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	Tissue Holder	
Y1 = Not used		
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used		
Max = Not used	Automatically generate an image of the whole sample area	
Image (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back	



j. Screenshot and save the live macro image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_US_macro.srf). Click Next

Pathology 1.0 (Alpha)		
Summary	Load sample	
Sample	Select sample type from the list below:	
Microscope slide 75 x 25 x 1 mm	Microscope slide 75 x 25 x 1 mm	
Image (macro)	Left CaF2 plate	
Not set	Right CaF2 plate	
Area of interest	Rotating holder	
X1 = Not used	◎ Tissue Holder	
Y1 = Not used	0	
X2 = Not used		
Y2 = Not used		
Surface		
Min = Not used	📼 Automatically, according to improve of the sub-the second	
Max = Not used	Automatically generate an image of the whole sample area	
lmage (hi-mag)	Go to load position Go to default position	
Not set	Restart Cancel < Back	

k. Using the annotated H&E images supplied by CENTRE 1, select the correct tissue section(s) to be used for Raman imaging on the newly generated macro montage.

Summary	Se	lect area	
		lectured	orinterest
Sample	Definition		
Microscope slide 75 x 25 x 1 mm	Image source	Video	
Image (macro)	Map type	Streamline (rect	angle)
None - use video	Region		
Area of interest	Start	E	nd Step
X1 = Not used	X -3258.3	426.1	1.1
Y1 = Not used	Y -1030.2	965.2	1.1
X2 = Not used			
Y2 = Not used			Binning 1
Surface			
Min = Not used	Points	3414	x 1815
Max = Not used			
Image (hi-mag)	Hi-mag montage size : 12 x 9		
Not set	Pertert	Cle	e Rack
	Sample Microscope slide 75 x 25 x 1 mm Image (maco) None - use video Area of intercat X1 = Not used Y1 = Not used Y2 = Not used Surface Min = Not used Image (hi-mag) Not set	Sample Definition Microscope slide Image source Image (macro) Mae type None - use video Region Area of interest X - 3258.3 Y1 = Not used Y - 1030.2 Y2 = Not used Y - 1030.2 Surface Min = Not used Max = Not used Points Image (hi-mag) Not set	Sample Definition Microscope slide 75 x 25 x1 mm Image source Image (macro) Map type Nore - use video Region Area of interest Start X1 = Not used Y Y2 = Not used Y Y2 = Not used Y Surface Max = Not used Max = Not used Points J414 Hi-mag montage Not set Restart

I. Deselect 'define surface' and click next

Pathology 1.0 (Alpha)		
Summary	Options	
Sample Microscope slide 75 x 25 x 1 mm Image (macro) Macro montage Area of interest X1 = 14,817.4 Y1 = -3,444.7 X2 = 15,898.5	 Define surface over selected region Collect image montage over selected region Let me review the montage before continuing Collect Raman map over selected region 	
Y2 = -2,671.6 Surface		
Min = Not used Max = Not used Image (hi-mag)		
Not set	Restart Cancel < Back Next >	

m. The workflow will prompt the user to verify focus of section at x25 magnification (you might have to switch between macro and x25 and move to different position to get representative area).



- n. Click next
- o. The workflow will begin creating the montage image of the selected section
- p. Once the montage has been created, Screenshot and save the image using Snipping tool (and Export -> Surface file -> save as ID_Path_Instit_US_section.srf)

Using Pathology workflow sub-module

 Once montage is completed, select 'refine the area of interest' from the next panel to redefine the area to be used for Raman mapping <u>FROM THE Hi-MAG</u> <u>IMAGE</u>. Click Next

Summary	Review hi-mag image	
Sample	Review the image and select the required action :	
Microscope slide 75 x 25 x 1 mm	Select a different area of interest	
mage (macro)	Create a surface to improve image focus	
Macro montage	Refine the area of interest	
Area of interest	Collect a Raman map	
X1 = 15,543.6		
Y1 = -2,494.8		
X2 = 15,657.0		
Y2 = -2,381.3		
Surface		
Min = Not used		
Max = Not used		
mage (hi-mag)		
Hi-mag montage	Restart Cancel < Back	
	-	
gy 1.0 (Alpha)		
C	Define area of interest	

Pathology 1.0 (Alpha)		8
Summary	Re	fine area of interest
Sample Microscope slide 75 x 25 x 1 mm Image (macro)	Definition Image source Map type	Hi-mag image : 'Microscope slide 75 x 25 x 1 rr • Streamline (rectangle) •
Macro montage	Region	
Area of interest		
X1 = 15,543.6		
Y1 = -2,494.8		
X2 = 15,657.0		No region selected.
Y2 = -2,381.3	Drag a rectar	ngle over the required area to select a region.
Surface	Alt-dra	ag a rectangle to zoom in on the image.
Min = Not used		
Max = Not used		
Image (hi-mag)		
Hi-mag montage	Restart	Clear < Back Next >

b. Using again the annotated H&E images supplied by CENTRE 1, select requested regions (ordered determined from CENTRE 1) on the live video image of the first section to image and click next



Pathology 1.0 (Alpha)			Ε	-1000 -	TT	T	1	T	TA	1 × 1	See.
Summary	Re	efine area of i	nterest		11	11					
Sample Microscope slide 75 x 25 x 1 mm Image (macro)	Definition Image source Map type	Hi-mag image : Micros Streamline (rectangle)	cope slide 75 x 25 x 1 π •		al	8	2	10			
Macro montage	Region				40.405	184	6.180		10000	10000	
Area of interest	Start	End	Step		SPREAS P	1.12	1 190	A . 2	AN MARCH	136 3	A. 1013.04/
X1 = 15,543.6	× 15498.0	15540.6	1.0	1	A DIAL OF THE	and the second	1000	2.2	100000000	Sector 10	
Y1 = -2,494.8	Y -2476.1	-2441.8	1.0		CONTRACTOR OF				81 S 866		2000000
X2 = 15,657.0				100		31 1.53	6.4.	Sec. 1	200		STREET, STREET
Y2 = -2,381.3			Binning 1			刻 色 調合	14.20	1000	1941	2.6	CONTRACTOR OF
Surface									1993	1000	100 C
Min = Not used						1000	1.2		19	5000	Constant of the local division of the local
Max = Not used				1.1		100		100	-	1000	1000
mage (hi-mag)	Points	42	x 34		025	A	E I	1.1	1.1	L	500 µm
Hi-mag montage	Restart	Clear	< Back Next >	1000	-5000 -25	536	-2000	-1500	-1000	-500	6

	Acquisition	File	Advanced	Uepth s	setup	vea serup	Surface	en ine image ar		
			TT	de la	Sim.		Suea	First	Last	Step
1	111	11		如云	all he	11	x	-477.0	313.4	10.4
+	++9						Y	19.6	862.0	10.4
1							Size	77 x 82	= 6314 po	ints
ß	2							se fived 7	0.0	YBin 10
100	COLUMN AND ADDRESS	Decision in the local division of the local	200	the set of	10 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	AND ST	103		0.0	
			a. 6					se med e	0.0	Slalom on
			0				Deta	ils	0.0	Slalom on 🗙
							Deta	ils • steps / map ste	р X 104.0 Y	Slalom on ★[-104.0
							Deta Stage Pre-s	ils e steps / map ste can spectra	p X 104.0 Y 9 spectra, 9	Slalom on -104.0 90 rows
							Deta Stage Pre-si CCD a	ils e steps / map ste can spectra area (l, r, t, b) sure & dwell	p X 104.0 Y 9 spectra, 9 X 10 1024, 6 s 6	Slalom on -104.0 90 rows Y 4.84 66.667
							Deta Stage Pre-se CCD a Expos	ils e steps / map ste can spectra area (l, r, t, b) sure & dwell er CCD pixel in Y	p X 104.0 Y 9 spectra, 9 X 10 1024, 6 s 6 1.04	Slalom on -104.0 90 rows Y 4 84 66.667

- c. **Optional**: Using the x25 in live viewer you can collect and save x25 images from area of interest.
- d. **IMPORTANT**: Switch to x25 magnification, focus position at area of interest on section and note down the Z focus position
- e. Clicking next will bring up the map measurement setup, click Range tab and set Laser name to fingerprint region

Range Acquisition File	e Advanced De	pth setup	Area setu	P			
StreamLine scan	Spectrum	Range					
Static	Low	Low -1381					
	Centre	520.0	0	Raman shift/cm-1 🔻			
	High	1848.	78				
Confocality	Configura	tion					
Standard	Laser name	2	Fingerprint	t 🗸			
(iii) High	Grating na	ne	1500 l/mm	•			
	Detector n	ame	Master: Re	enishaw CCD Camera 🔹			
	Calibration	Calibration status 🖌 Calibrated OK					

f. Click acquisition tab and set exposure time to <u>6s</u>.

ange Acquis	ition File Advanced Depth se	etup Area setup	
Exposure time	/s 6.00 🚔 Objective	50	Cosmic ray removal
Accumulations	1 Laser power / %	. 100 -	Response calibration
Live imaging			Restore instrument state on completion
		New	Close laser shutter on completion
Not using live	e imaging.	Edit LUT	Minimize laser exposure on sample
Title	'Microscope slide 75 x 25 x 1 mm'	loaded @ 16:39 on 20/05/2016 / From (154	98.0, -2476.1) to (15540.6, -2441.
Description			
Description	amLine image measurement created	by the Streamline image acquisition wizard	

- g. Click area setup tab and make sure YBin is set to <u>10</u> (step size should default to ~10.4um for X and Y) and check slalom on.
- h. Check the 'Use fixed Z' checkbox and enter the value noted from step d

Str	reamLine image area											
	Fire	st			I	.ast		Step				
x	þ15	498.	0			16111.6		10.4				
Y	-2	476.	1		[-1862.5		10.4				
Size	e 60) x 60				= 3600 points						
	Use fixed Z			[0.0	YBin	10					
						₹?						
Det	Details											
Stage steps / map step)	X 104.0 Y	′ -104	4.0					
Pre-scan spectra				9 spectra,	90 ro	ows						
CCD area (l, r, t, b)				X 10 1024,	Y 4	84						
Exposure & dwell				6 s 666.667								
µm j	per C	CD p	ixel	in Y		1.04						
Dur	ation					2766.0 s						
	Strea	amLine	e imi	age ar	ea							
		First				Last		Step				
	X	154	98.0)		16111.6		10.4				
	Y	-247	76.1			-1862.5						
	Size	60		x 60		= 3600 po	ints					
	V V	se fix	ed Z	!		5]	YBin	10			
		4					Sla	lom on 🛛	✓ ?			
	Deta	IIS	- 1-			V 104 0 V	104	0				
	Dra	step	5 / I	nap sti	гþ	A 104.0 Y	-104.	.0				
	CCD	can sj area (bect 1 r	th)		y specula,	9010\ Y49	4				
	Expos	sure 8	k dw	ell		6s 6	666,66	57				
	µm pe	er CCI	D pi	kel in Y	,	1.04						
	Durat	ion				2766.0 s						

- i. Click apply and then OK
- j. Run a PQ before starting Raman map measurement and ensure that PQ passes.



- k. Click Run measurement to begin Raman map measurement
- I. When map measurement is complete, run a PMC to make sure there is no shift in wavenumber and spectral intensity from PQ has occurred

To create additional Raman maps

- a. **IMPORTANT**: Switch to x25 magnification, focus position at area of interest on section and note down the Z focus position
- b. To begin setting up Raman map from existing Hi-Res image, the user will need to make sure the correct montage is selected from the Window menu
- c. User will click on Measurement->New->Streamline image acquisition



- d. Using again the annotated H&E images supplied by CENTRE 1, select requested regions (ordered determined from CENTRE 1) on the <u>FROM THE Hi-MAG IMAGE</u> just created
- e. NB: remember to select 'Active Window' as the image source in the panel)

	StreamLine image area sele	ection		1	
	StreamLine image area		ОК		
	Please draw an ar	rea onto the video/image	Cancel		
	Details				
	Image source Video viewer Active window				
Reflected	8 <u>H</u>	mag image : Microscope slide 73 x 23 x 1	mm' loaded @ 14:37 on 30/03/2018	/ From (-1000-2, -1181.9) to @77.9,	999.5)
Pathology 1.0 (Alpha)	* (1	1 Hi-mag image : Microscope slide 75 x 2	5 x 1 mm ⁻ loaded © 15:34 on 30/03/2	2016 / From (-3258.3, -1030.2) to (4	26.1, 965.2)
Summary	Select region			10000	
Sample Microscope slide 75 x 25 x 1 mm Image (macm)	Definition Image source [Himag image 1: Monscope side 75 x 25 x 1 n •] Map type [Sesamine Incluring] •]	100	A.	ATY	
Area of interest XT = -671.2 YT = -995.1 X7 = 671.2	Sant End Step X 4770 311.1 1.1 Y 156 156.4 1.1				
Y2 = 72.1 Surface Min = .0	Brining 1	500 -	C AND	C. T. C.	
Max = 4 Image (hi-mag) Hi-mag montage	Pointa 721 x 778	1000		TE	[530 µm]
	Fester Clear clack Nexts	-3000 -2500	-2000 -1500	-1000 -500	¢

- f. **Optional**: Using the x25 in live viewer you can collect and save x25 images from area of interest.
- g. Clicking next will bring up the map measurement setup, click Range tab and set Laser name to fingerprint region

o measurement setup							8	23
Range Acquisition File	Advanced Dep	th setup	Area	setup				
StreamLine scan	Spectrum R	ange						
Static	Low	Low -1381						
	Centre 520.		0	Raman shift	t/cm-1	•		
	High	1848.	78					
Confocality	Configuratio	on						
Standard	Laser name		Finge	print			·	
High	Grating nam	Grating name		/mm		-	·	
	Detector nar	me	Maste	r: Renishaw CCD	Camera	-	•	
	Calibration s	Calibration status		Calibrated OK				
				ОК	Cancel	Apply	He	lp

h. Click acquisition tab and set exposure time to 6s

Exposure time	/s 6.00	Objective	50	Cosmic ray removal
Accumulations	1	Laser power / %	100 🔻	Response calibration
Live imaging				Restore instrument state on completion
			New	Close laser shutter on completion
Not using liv	e imaging.		Edit LUT	Minimize laser exposur on sample
Title	'Microscope s	slide 75 x 25 x 1 mm' loa	aded @ 16:39 on 20/05/2016 / From	(15498.0, -2476.1) to (15540.6, -2441
Description				
Description	aml ine image m	easurement created by	the Streamline image acquisition wize	ard
This is a Stre	antene inage in			
This is a Stre	anene mage m			

- i. Click area 'Area setup tab' and make sure YBin is set to <u>10</u> (step size should default to ~10.4um for X and Y) and check sialon on.
- j. Check the 'Use fixed Z' checkbox and enter the value noted from step b

Strea	mLine image area						
	First	Last	Ste	2p			
X	15498.0	16111.6	10	.4			
<u>Y</u>	-2476.1	-1862.5	þ10	<u>10.4</u>			
Size	60 x 60	= 3600 poi	nts				
V Us	se fixed Z	5	YB	in 10			
			Slalor	non 📝 ?)		
Detai	ls						
Stage	steps / map step	X 104.0 Y -104.0					
Pre-so	can spectra	9 spectra, 90 rows					
CCD a	area (l, r, t, b)	X 10 1024,	X 10 1024, Y 4 84				
Expos	ure & dwell	6s 6	6 s 666.667				
µm pe	er CCD pixel in Y	1.04					
Durat	ion	2766.0 s					

The map parameters of each of the Raman maps can be saved/captured using the Snip-tool



windows function to grab the Area setup panel (

- k. Click apply and then OK
- I. Run a PQ before starting Raman map measurement and ensure that PQ passes.



- m. Click Run measurement to begin Raman map measurement
- n. When map measurement is complete, run a PMC to make sure the no shift in wavenumber and spectral intensity from PQ has occurred

D. Raman Measurement collection

a. Repeat above process for the other areas on the section and slide references from the annotated H&E images

Eventually you will have a collection of Raman maps from the selected areas:



E.g. different region on section





E.g. different section on slide





- b. Remember to shutdown Wire before starting a new setup of Raman measurements due to memory leak issues with current version
- c. Repeat this process (Imaging and selection of areas of Interest from unstained sections using H&E annotated images) for the other tissue slides using the annotated H&E images





