



Suggested protocol to use R221 from CryoCapCell

1- General considerations about R221 resin

In the following document, we propose one use of R221 resin. Since this product is still being largely explored by the scientific community, we recommend that you keep yourself posted to see suggested developments of the products use, either by communicating with CryoCapCell or with other scientists that might have tested the product and adapted the use to their own projects.

R221 resin is a methacrylate based acrylic resin that can be used in comparable fashion to Lowicryl HM20 with slight adaptation with regard to the temperatures.

R221 resin is preferentially dissolved in 1- Ethanol or 2- Acetone.

R221 resin remains fluid at -30°C and becomes more viscous below this temperature.

In solution with Ethanol or Acetone, (up to 90% resin, 10% solvent), it remains fluid until -45°C. Therefore, we recommend adapting your embedding protocol to these parameters to get optimal embedding quality.

We recommend using a freeze substitution automate to get reproducible results (any available from the market).

2- R221 storage

R221 resin is a methacrylate based resin. Therefore, you should follow your institution's guidelines on storage of methacrylate products.

If no specific recommendations are edicted by your host institution, the R221 can be safely stored at room temperature or between 4 and 8°C, in a fume cabinet for 12 month, must remain protected from light.

Storage at -20°C or lower is not necessary.

3- Timelines for FS protocols

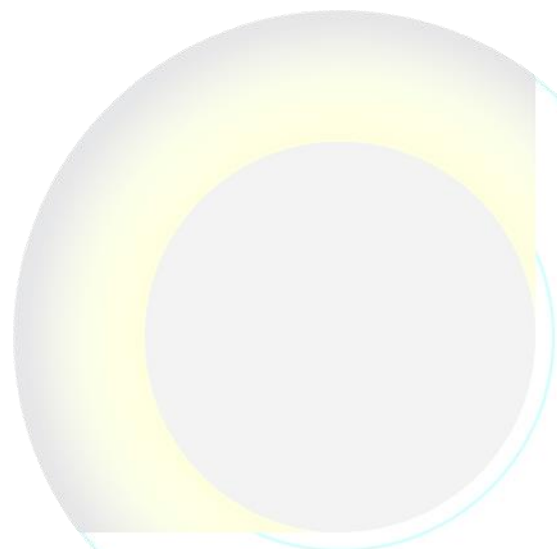
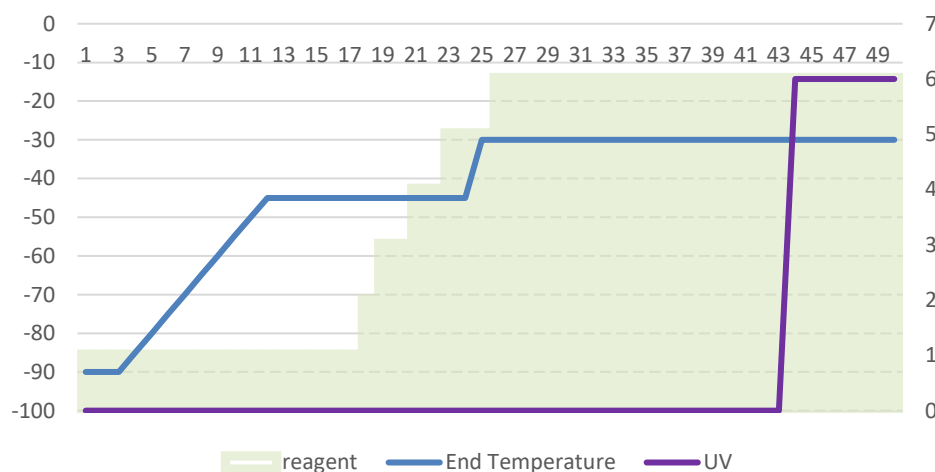
The following table is one protocol we commonly apply to embed High Pressure Frozen material.

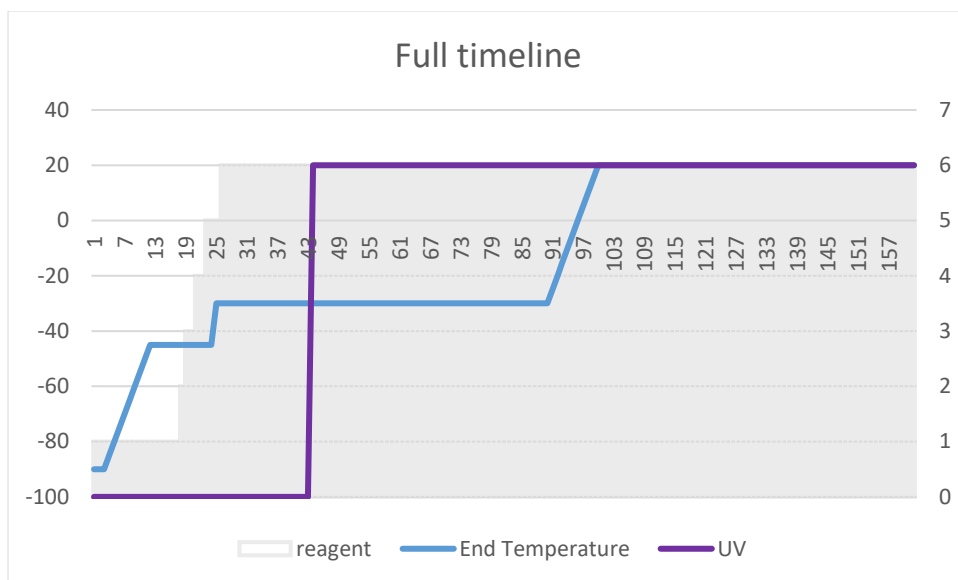
The lines **highlighted in yellow** should be adapted to your own agenda and represent steps usually planned overnight.

The infiltration steps are important and **should not be reduced in steps or length**, but can be increased upon sample requirement (bulk specimens, long-term infiltration requirements etc).

Step	Start Temperature	End Temperature	Slope	Day	time (hh:mm)	Reagent	Concentration	Transfer	Agitation	UV	Pause
1	-90	-90	0	1	overnight	FS CLEM	100%	stay	off		
2	-90	-45	5		09:00	FS CLEM	100%	stay	off		
3	-45	-45	0	2	05:00	FS CLEM	100%	stay	off		
4	-45	-45	0		00:10	Aceton	100%	exch/fill	off		
5	-45	-45	0		00:10	Aceton	100%	exch/fill	off		
6	-45	-45	0		00:10	Aceton	100%	exch/fill	off		
7	-45	-45	0		02:00	R221	25%	exch/fill	ON		
8	-45	-45	0		02:00	R221	50%	exch/fill	ON		
9	-45	-45	0		02:00	R221	75%	exch/fill	ON		
10	-45	-30	30		00:30	R221	75%	stay	ON		
11	-30	-30	0		overnight	R221	100%	exch/fill	off		
12	-30	-30	0	3	02:00	R221	100%	exch/fill	ON		
13	-30	-30	0	5	48:00:00	R221	100%	stay	off	X	
14	-30	20	5	6	10:00	R221	100%	stay	off	X	
15	20	20	0	7	48:00:00	R221	100%	stay	off	X	

FS Timeline : 0h to begining of UV





4- Polymerization and storage conditions

R221 resin requires ultra-violet light and low oxygen levels to polymerize properly. Make sure your freeze substitution instrumentation contains enough liquid nitrogen to complete the polymerization cycle.

Polymerization of the resin at -30°C should not be shorter than 24hours before raising the temperature to room temperature, still under UV. After reaching room temperature, it is possible to transfer the specimen blocks in a separate UV box to free the freeze substitution machine. An extra 24hours of UV exposure is recommended to ensure all the resin has reacted properly and limit evaporation of acrylate residues.

After polymerization, the specimen blocks can be stored at room temperature, protected from light if fluorescence must be preserved. Storage at +4°C is possible but not mandatory.

5- Freeze substitution protocols

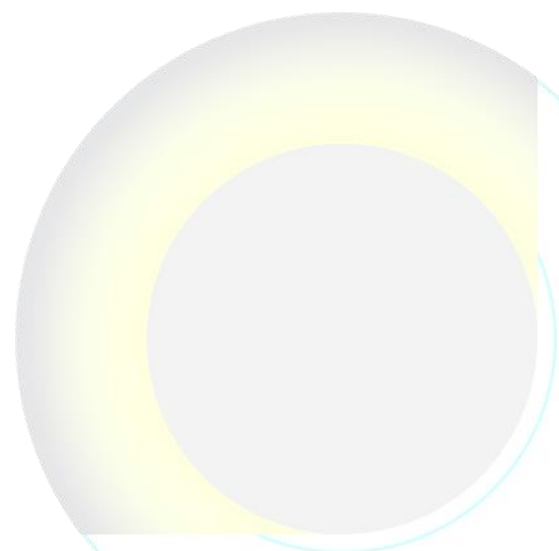
The freeze substitution cocktail is flexible; however, we **do not notice any improvement of the sample contrast with Uranyl Acetate concentrations higher than 1%.**

The following FS cocktails have been tested, which gave satisfactory results in terms of contrast and ultrastructure. Alternative cocktails may be tested according to your own protocols

Cocktail for Fluorescence preservation					
	FS 1	FS 2	FS 3	FS 4	Solution
Concentration (in %)	0.05	0.01	0.01	0.10	Uranyl Acetate
	0.01	0.00	0.01	0.50	Glutaraldehyde
	5.00	0.05	5.00	1.00	H2O
	94.94	99.94	94.98	98.40	Acetone
	0.00	0.00	0.00	0.00	Ethanol

We are not aware of contrasting agents that could alter the embedding and polymerization properties of the resin, however, it is generally not recommended to use Osmium tetroxide (OsO₄) since the dark precipitate created in the presence of oxygen tends to prevent UV penetration and the resin cross-linking.

Oxygen should also be avoided to ensure proper polymerization of the resin (CO₂, or N₂ saturated environment).



6- Progressive Lowering Temperature (PLT) protocol

Start temp	End temp.	slope	Duration	Solution	UV
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Impregnation with heavy metal salts

+4°C				30% ethanol + uranyl acetate 2% in H ₂ O	
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Adjust timing according to the sample thickness. If too large, place on wheel overnight at 4°C.

Dehydration

program PLT-1

+4°	+4°	0	0h30	50% ethanol	no
+4°	+1°	-15	0h12	50% ethanol	no
+1°	+1°	0	0h30	70% ethanol	no
+1°	-20°	-30	0h30	70% ethanol	no
-20°	-20°	0	0h30	80% ethanol	no
-20°	-35°	-30	0h30	90% ethanol	no
-35°	-35°		0h30	100 % ethanol 3 x 10 min	no

0,3 = 30 min on AFS2 program

0,12 = 12 min

49h

Infiltration

-35°	-35°		2h	R221 25% in ethanol	no
-35°	-35°		16h30	R221 50%	no
-35°	-35°		6h	R221 75%	no
-35°	-35°		24h	R221 100%	no

Split samples in 2 groups for polymerization

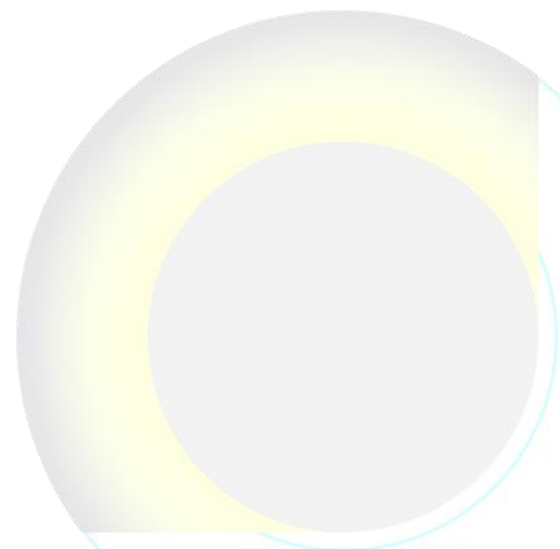
Polymerization

program PLT-2

-30°	-30°	0	48h	UV (48h -30°)	yes
-30°	-20°	2°C/h	5h		yes
-20°	-20°	0	8h		yes
-20°	+20°	5°C/h	8h		yes
+20°	+20°	0	20h		yes

1) In resin blocks

2) Naked for minimal resin embedding protocol (Schieber et al, 2017)



7- Finding the right parameter to image the resin

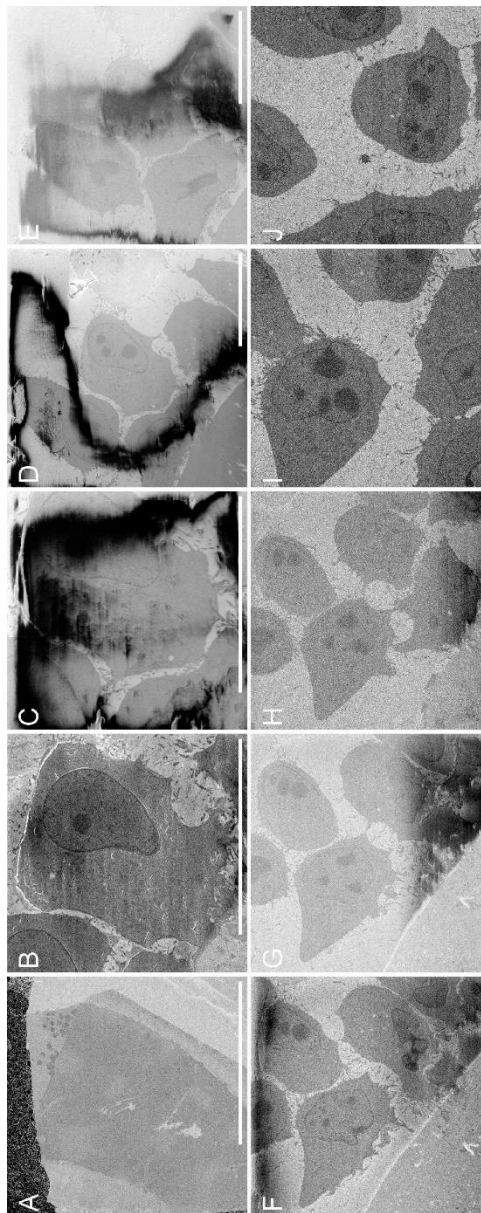
Microscope: Zeiss Sigma VP with Gatan 3View XP2

Sample prep: Block sent to Chris Peddie was divided into two pieces. One piece was mounted onto an aluminium pin using conductive epoxy glue and baked over the weekend. Trimmed edges, noticed that resin is very brittle.

Coating: None

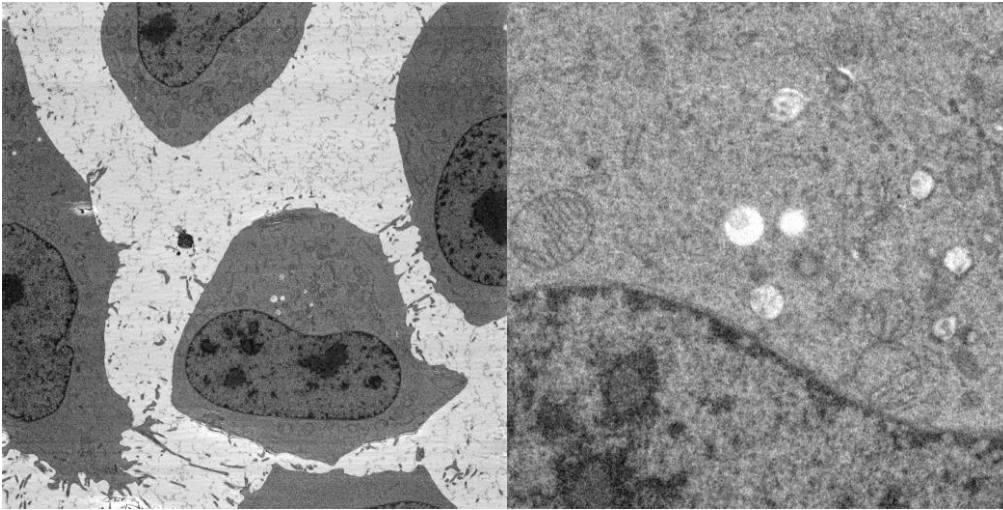
The arrow in A points toward the region used to define the imaging parameters. At the top of the frame, the silver-epoxy mounting glue is visible. The overview illustrate the absence of coating prior to imaging.

Voltage:		High Current:		Aperture:		Pressure:		Magnification:		Contrast:		Frame size:		Pixel size:		Dwell time:		Slice thickness:		No. of slices:		Comments:	
1	2 kV	Off	30 micron	10 Pa (N2 gas)																		No significant charging when imaging and focusing during set up. Contrast on cells is low. Surface features ('shattering of resin) during cutting at 100 nm. Difficult to focus and stigmatize due to low contrast on cells.	
3	2 kV	Off	30 micron	10 Pa (N2 gas)	10593	8	5	2048x2048	12 nm	4 us	50 nm	21										Organelles visible but very low contrast in cytoplasm. Some resin damage in the form of smearing perpendicular to knife edge. Inconsistent cutting	
4	2 kV	Off	30 micron	HV	10593	8	5	2048x2048	12 nm	4 us	50 nm	6										Increase contrast by moving to high vacuum. Severe charging across some areas, but possible to focus on cells due to increased contrast in image. Run attempts – severe charging and image shifts, resin damage. Aborted after 5 images.	
5	2 kV	Off	30 micron	HV	5000	8	5	2048x2048	25 nm	4 us	50 nm	6										Reduced magnification to counteract charging and dose effects. Charging and image deformation still severe. Aborted after 5 images.	
6	2 kV	Off	20 micron	HV	5000	8	5	2048x2048	25 nm	4 us	50 nm	6										Smaller aperture to counteract charging and dose effects. Low contrast, difficult to focus and stigmatize. Severe charging and image movement. Aborted after 5 images.	
7	2 kV	Off	30 micron	6 Pa (N2 gas)	5000	8	5	2048x2048	25 nm	4 us	50 nm	11										Returned to VP. 5 Pa to counteract charging. Had to return to 30 micron aperture as signal too low to focus. Low contrast, poor organelle recognition. Increased pressure during at slice 7 to 6 Pa, improved charging slightly but images still poor.	
8	2 kV	Off	30 micron	6 Pa (N2 gas)	5000	8	5	4096x4096	13 nm	4 us	50 nm	9										Increased frame size to improve pixel resolution. Charging better but still present in some regions of image. Cutting better but inconsistent. Some image deformation present. Poor contrast on cytoplasm. Pressure increased to 8 Pa during acquisition of slice 4, improved charging but decreased contrast.	
9	2 kV	Off	30 micron	8 Pa (N2 gas)	5000	8	5	8192x8192	13 nm	2 us	50 nm	12										Increased frame size to improve resolution, decreased dwell time to maintain dose.	
10	2 kV	Off	30 micron	8 Pa (N2 gas)	7500	8	5	8192x8192	4.3 nm	3 us	100 nm	17										Increased mag and dwell time to improve contrast and resolution. Stable run, resolution OK, contrast average. No visible charging. Consistent cutting.	
11	2 kV	Off	30 micron	8 Pa (N2 gas)	8000	8	5	8192x8192	4 nm	3 us	100 nm	116										Increased mag to improve resolution.	



Final conditions on the Sigma VP SEM (Zeiss), the Crick Institute

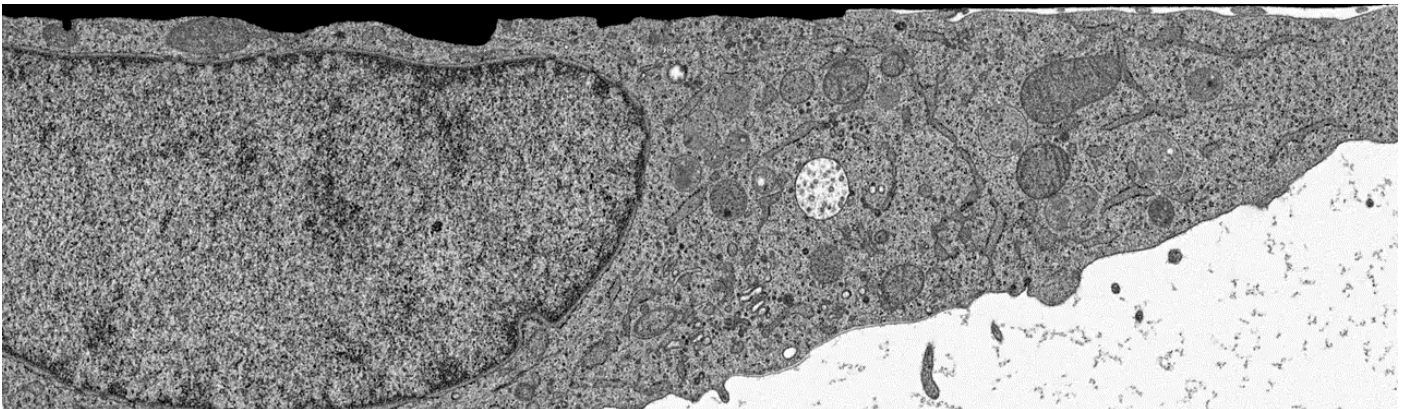
Voltage: 2 kV; High Current: Off; Aperture: 30 micron; Pressure: 8 Pa (N2 gas); Magnification: 7500; Contrast: 8; Brightness: 5; Frame size: 8192x8192; Pixel size: 4.3 nm; Dwell time: 3 μ s. Slice thickness: 100 nm - 3View2XP (Gatan)



Zeiss Crossbeam 540 SEM

Accelerating voltage: 1.5 keV; Current: 1 nA; EsB detector (1200 V grid); Frame size: 4139x1549 (rotated); Pixel size: 5 nm; Dwell time: 10 μ s.

Slice thickness: 5 nm; Atlas 5 for 3D tomography acquisition (Zeiss)

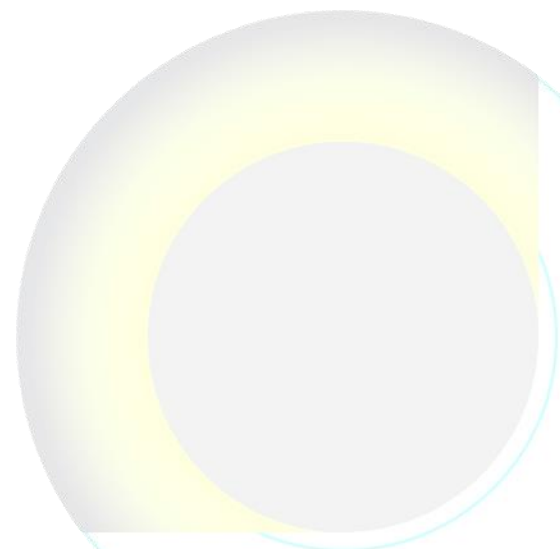


ThermoScientific volumeScope

Pressure chamber: 40Pa

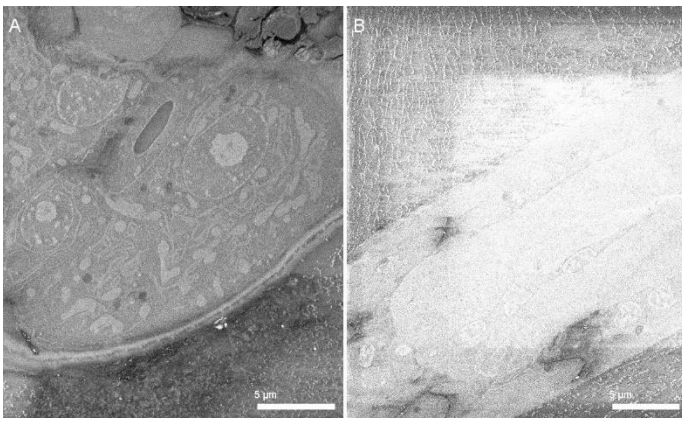
Accelerating voltage: 2keV; Current: 200pA; detector: BSD; Pixel size: 10nm; Dwell Time : 3 μ s

Slice thickness: 65nm



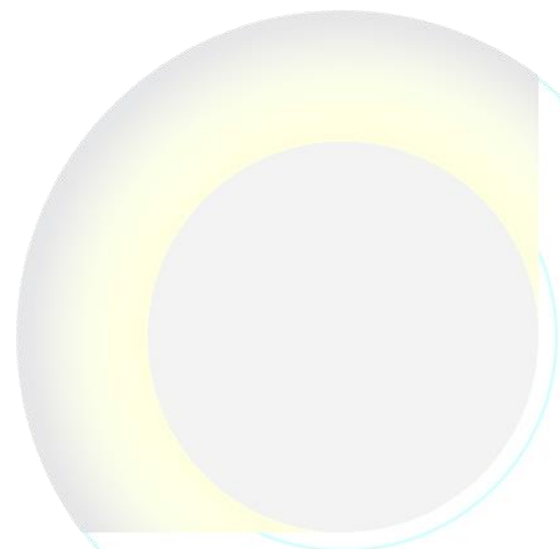


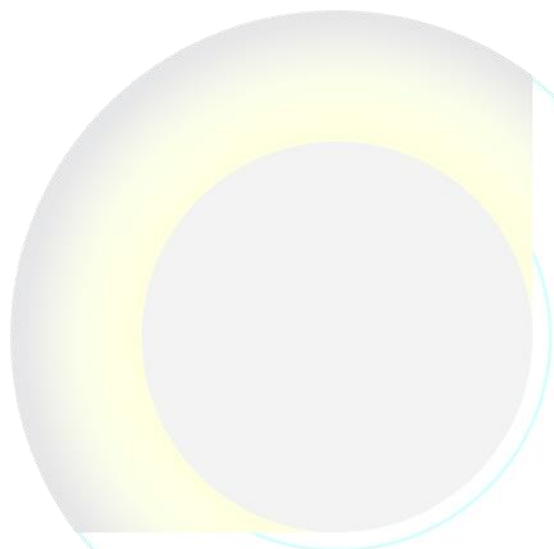
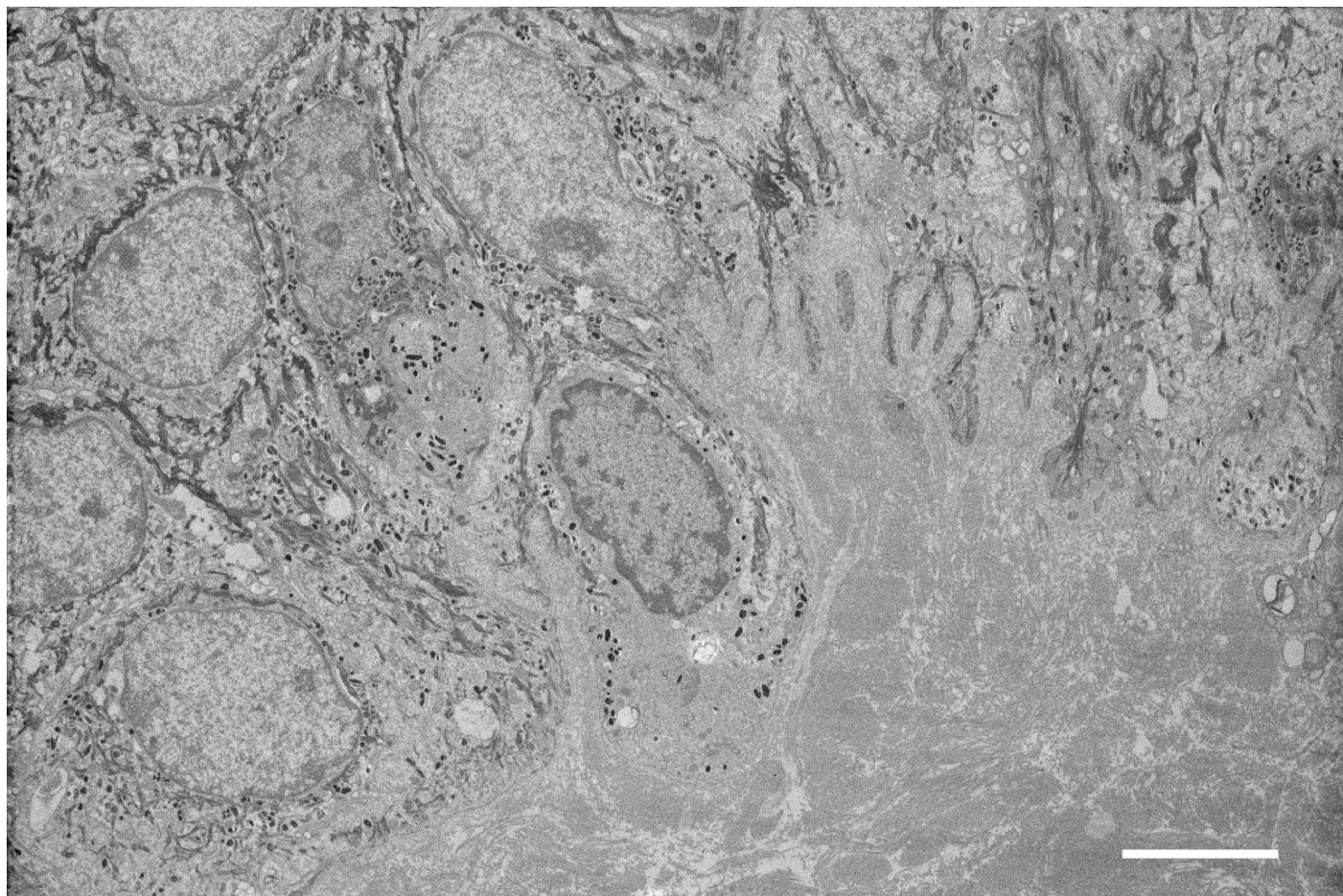
Imaging condition comparison



Comparative charging effect of an embedded *C.elegans* between R221 resin (A) and conventional HM20 (B). In this freeze substitution protocol, only 0.01% uranyl acetate and 5% H₂O in dry acetone were used.

PLT prepared sample





SAFETY DATA SHEET (453/2010/EU)

PRODUCT Date : 19/06/18 HYB-221 Version 1

1. IDENTIFICATION OF THE SUBSTANCE/ MIXTURE AND OF THE COMPANY/ UNDERTAKING

1.1 PRODUCT IDENTIFIER : HYB-221

1.2 RELEVANT IDENTIFIED USES OF THE SUBSTANCE OR MIXTURE AND USES ADVISED AGAINST :
Photoreticulable monomers

2. HAZARDS IDENTIFICATION

2.1 CLASSIFICATION OF THE SUBSTANCE OR MIXTURE :

CLASSIFICATION ACCORDING TO THE REGULATION N° 1272/2008/EC :

Skin Irrit. 2 (H315) - Causes skin irritation

Skin Sens. 1 (H317) - May cause an allergic skin reaction.

Eye Dam. 1 (H318) - Causes serious eye damage

STOT SE 3 (H335) - May cause respiratory irritation

2.2 LABEL ELEMENT :

This product is labelled according to the Regulation n° 1272/2008/EC :

Signal word : Danger

Symbol :



Hazard statement :

H315 Causes skin irritation

H317 May cause an allergic reaction.

H318 Causes serious eye damage

H335 May cause respiratory irritation

Precautionary statement :

Prevention :

P261 Avoid breathing vapours

P280 Wear protective gloves/protective clothing/eye protection/face protection.

Response :

P305 + P351 + P338 If in eyes : rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.

Hazardous components which must be listed on the label : Acrylates.

2.3 OTHER HAZARDS :

None.

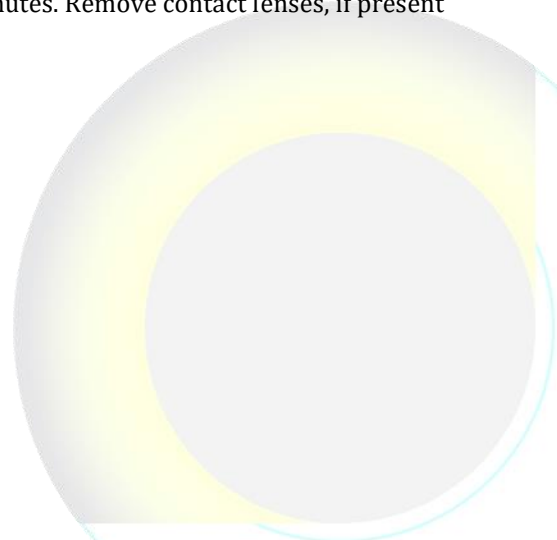
3. COMPOSITION/ INFORMATION ON INGREDIENTS

Product nature : Mixture

contact@cryocapcell.com

www.cryocapcell.com

0033-6-51-05-46-62



Substance	%	N° EC	REACH	Classification (1272/2008/CE)
Acrylates	> 50	203-652-6	*	Skin Irrit. 2 (H315) Eye Irrit. 2 (H319) STOT SE 3 (H335)
Acrylates	> 10	[CAS] 524969-08-9	*	Skin Irrit. 2 (H315) Skin Sens. 1 (H317) Eye Dam. 1 (H318)
Ether	< 1	[CAS 3524-62-7]	*	Acute Tox. 3 (H301)

*A registration number is not available for this substance as the substance or its uses are exempted from registration, the annual tonnage does not require a registration or the registration is envisaged for a later registration deadline.

4. FIRST AID MEASURES

4.1 DESCRIPTION OF FIRST AID MEASURES :

INHALATION : If breathed in, move person into fresh air. Consult a physician

EYES CONTACT : Rinse with plenty of water, also under the eyelids, for at least 15 minutes.

In the event of persistent irritation or any lesions, consult an oculist.

SKIN CONTACT : Wash off with soap and water.

INGESTION : Clean mouth with water. Do not induce vomiting.

4.2 MOST IMPORTANT SYMPTOMS AND EFFECTS, BOTH ACUTE AND DELAYED :

No data available.

INDICATION OF ANY IMMEDIATE MEDICAL ATTENTION AND SPECIAL TREATMENT NEEDED :

No data available.

5. FIREFIGHTING MEASURES

5.1 EXTINGUISHING MEDIA :

SUITABLE EXTINGUISHING MEDIA : Use water spray, alcohol-resistant foam, dry chemical or carbon dioxide

UNSUITABLE EXTINGUISHING MEDIA : High pressure water

5.2 SPECIAL HAZARDS ARISING FROM THE SUBSTANCE OR MIXTURE :

Carbon oxides

5.3 ADVICE FOR FIREFIGHTERS : Wear self contained breathing apparatus for the fire fighting if necessary.

6. ENVIRONMENTAL PRECAUTIONS

6.1 PERSONAL PRECAUTIONS, PROTECTIVE EQUIPMENT AND EMERGENCY PROCEDURES :

FOR NON-EMERGENCY PERSONNEL : Suitable gloves, protective glasses, protective clothing.

FOR EMERGENCY PERSONNEL : Self-contained breathing apparatus.

6.2 ENVIRONMENTAL PRECAUTIONS :

Do not let product enter drains

6.3 METHODS AND MATERIAL FOR CONTAINMENT AND CLEANING UP :

Absorb with mineral filler (such as sand, diatomaceous earth,...), collect and store in a suitable container.

6.4 REFERENCE TO OTHER SECTION

contact@cryocapcell.com

www.cryocapcell.com

0033-6-51-05-46-62

7. HANDLING AND STORAGE

7.1 PRECAUTIONS FOR SAFE HANDLING :

Avoid contact with skin and eyes. Avoid inhalation of vapour.

7.2 CONDITIONS FOR SAFE STORAGE, INCLUDING ANY INCOMPATIBILITIES :

Store between 2 and 8 °C (recommended) in a sealed containers (dark room).

7.3 SPECIFIC END USE(S) :

No data available.

8. EXPOSURE CONTROLS/ PERSONAL PROTECTION

8.1 CONTROL PARAMETERS :

None.

8.2 EXPOSURE CONTROLS :

8.2.1 Appropriate engineering controls :

Eye/ face protection : Safety glasses

Hand protection : Gloves.

Respiratory protection : Use an appropriate equipment NIOSH (US) or EN166 (EU)

Thermal hazards : Not concerned.

8.2.2 Environmental exposure controls :

No data available.

9. PHYSICAL AND CHEMICAL PROPERTIES

9.1 INFORMATION ON BASIC PHYSICAL AND CHEMICAL PROPERTIES :

Appearance : Liquid Odour : No data available

Odour threshold : No data available pH : No data available

Melting point : No data available Relative density : No data available

Boiling range/ initial boiling point : 170°C Solubility(ies) : No data available

Flash point : 113°C Coefficient n-octanol/ water : 1.88

Flammability : Not concerned Viscosity : Liquid

Explosive properties : No data available Vapour density : Not concerned

Oxidising properties : No data available Evaporation rate : Not concerned

Vapour pressure : Not concerned Auto-ignition temperature : Not concerned

Explosive limits : Not data available Decomposition temperature : No data available

9.2 OTHER INFORMATION :

None.

10. STABILITY AND REACTIVITY

10.1 REACTIVITY :

No data available.

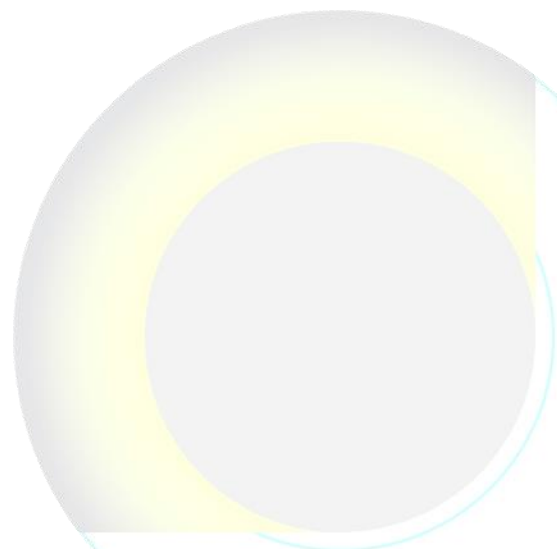
10.2 CHEMICAL STABILITY :

Contains the following stabilisers : Mequinol 100 ppm and BHT 20 ppm.

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10.3 POSSIBILITY OF HAZARDOUS REACTIONS :

No data available.

10.4 CONDITIONS TO AVOID : High temperature and UV-light

10.5 INCOMPATIBLE MATERIALS : Strong bases, strong oxidizing agents.

10.6 HAZARDOUS DECOMPOSITION PRODUCTS : Carbon oxides.

11. TOXICOLOGICAL INFORMATION

11.1 INHALATION : No data available.

11.2 INGESTION : DL50 Oral – rat -10.000 mg/kg

11.3 SKIN CONTACT : May cause an allergic reaction

11.4 EYES CONTACT : The product causes irritation of eyes.

12. ECOLOGICAL INFORMATION

In normal use conditions, this product may be released into water.

12.1 TOXICITY : No data available.

12.2 PERSISTENCE AND DEGRADABILITY : No data available.

12.3 BIOACCUMULATIVE POTENTIAL : No data available.

12.4 MOBILITY IN SOIL : No data available.

12.5 RESULTS OF PBT AND VPVB ASSESSMENT : PBt/vPvB assessment not available

12.6 OTHER ADVERSE EFFECTS : No data available.

13. DISPOSAL CONSIDERATIONS

This product may be disposed of under controlled incineration and in agreement with local and national legislation.

14. TRANSPORT INFORMATION

14.1 UN NUMBER : ADR/RID : - IMDG : - IATA : 3334.

14.2 UN PROPER SHIPPING NAME : ADR/RID : - IMDG : - IATA : Aviation regulated liquid, n.o.s

14.3 TRANSPORT HAZARD CLASS(ES) : ADR/RID : - IMDG : - IATA : 9.

14.4 PACKING GROUP : ADR/RID : - IMDG : - IATA : III.

14.5 ENVIRONMENTAL HAZARD : No

14.6 SPECIAL PRECAUTIONS FOR USER :

15. REGULATORY INFORMATION

The classification of the product has been realised according to the Regulation n°453/2010/EU and with the Directive n°67/548/EC.

15.1 SAFETY, HEALTH AND ENVIRONMENTAL REGULATION/ LEGISLATION SPECIFIC FOR THE SUBSTANCE OR MIXTURE :

contact@cryocapcell.com

www.cryocapcell.com

0033-6-51-05-46-62

Not concerned.

15.2 CHEMICAL SAFETY ASSESSMENT : Not realized.

16. OTHER INFORMATION

This material data sheet complements, but does not replace our Technical Data Sheet. The information is, to the best of our knowledge true and accurate,

but any recommendations or suggestions which may be made are without guarantee, since the conditions of use are beyond our controls. For a revised Safety Data

Sheet the information, which has been added, deleted or revised, is noted with (*)

