Pre Fixation Artefacts

Who? Amanda Griffin, I am the EM lead, H&S manager and Advanced BMS

Where? I have worked most of my career at the RVI, Newcastle upon Tyne (2yrs Conquest Hospital in 1996)

Why? I began in routine Histopathology in 1996, interested in EM when moved to RVI, started training in EM in 2006, took over as lead in 2018.

What? We are a referral centre for renal services in the North East of England.

The variation we see in our biopsies is massive, but we don't really know where this variation comes from

– all biopsies are different, but how different?

Does the treatment of the biopsy we receive really have that much of an impact on what we see down the microscope?

Biopsies come to us in one of 6 different ways, we will try all to see the effects they have on our tissue, average travelling time is 3 hours so we started there…

Kidney cores from background of RCC nephrectomy



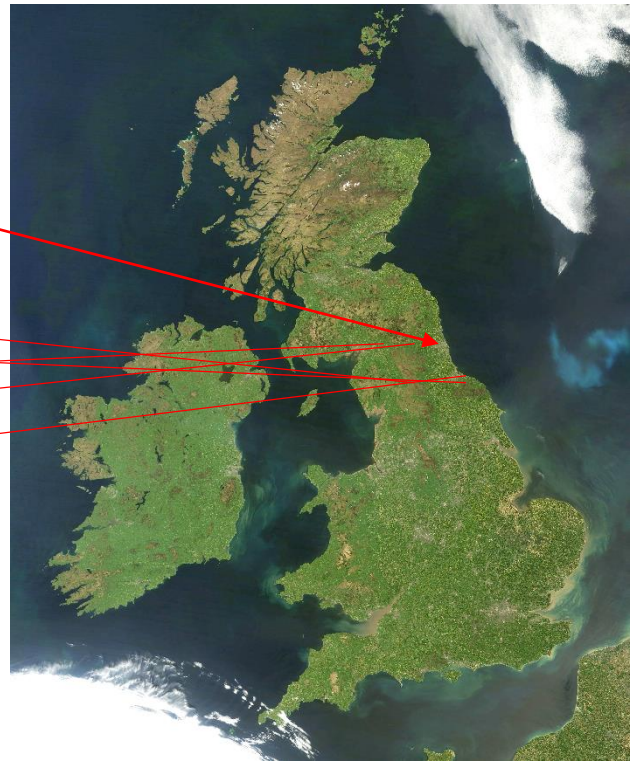
Organ removed approximately 2 hours prior to cores taken, kept in a fridge, cores removed using renal core needle from theatres on the dissection bench.

Rest of the procedure is as routine renal protocol except for the pre-exam phase which was altered for the trial.

All of our standard solutions and equipment used – details at the end if you'd like them

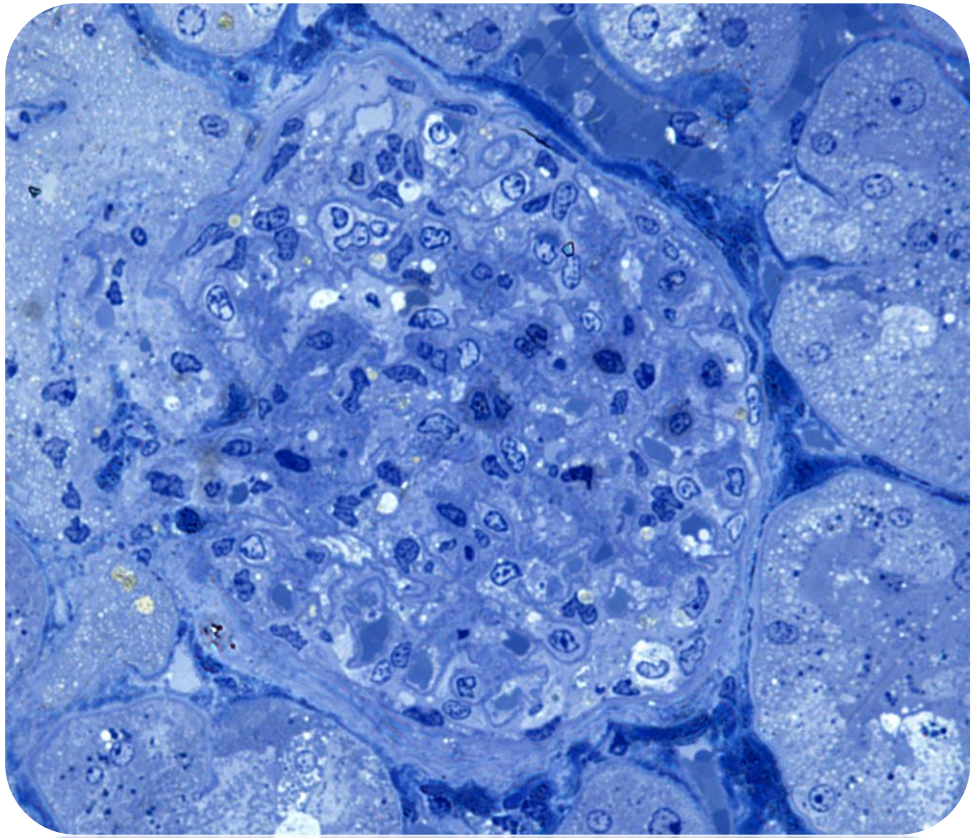
Variations : 3 Hours at trial solution then transferred into 2.5% glute (with the exception of the routine - Sucrose / Sorensen's) We looked at how and where our cases were coming from – what sort of variables would there be? What had we received in the past?

- Routine 2.5% glutaraldehyde at 5°C (all in house)
- Room temperature Glutaraldehyde
- Formalin
- Left dry
- Michel's medium
- Pure / RO water
- XS PBS
- Long term storage post fixation

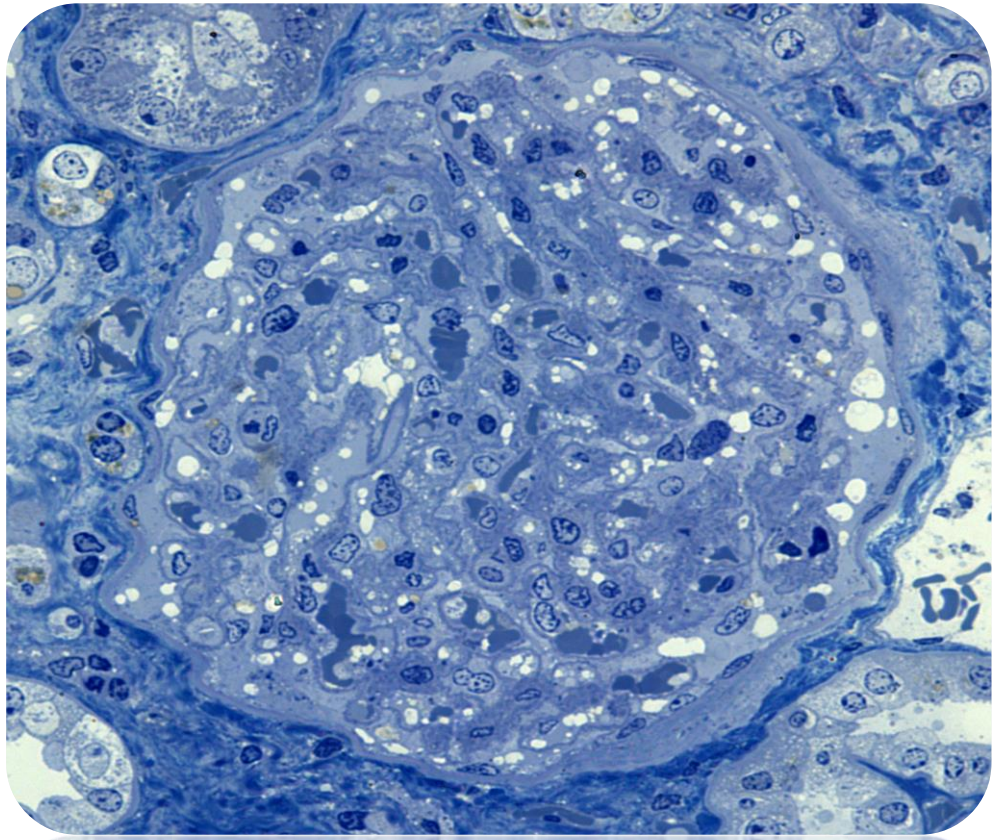


So… Let’s Compare

Left side will always be our routine processes, the right side will be the changed process to see how they differ

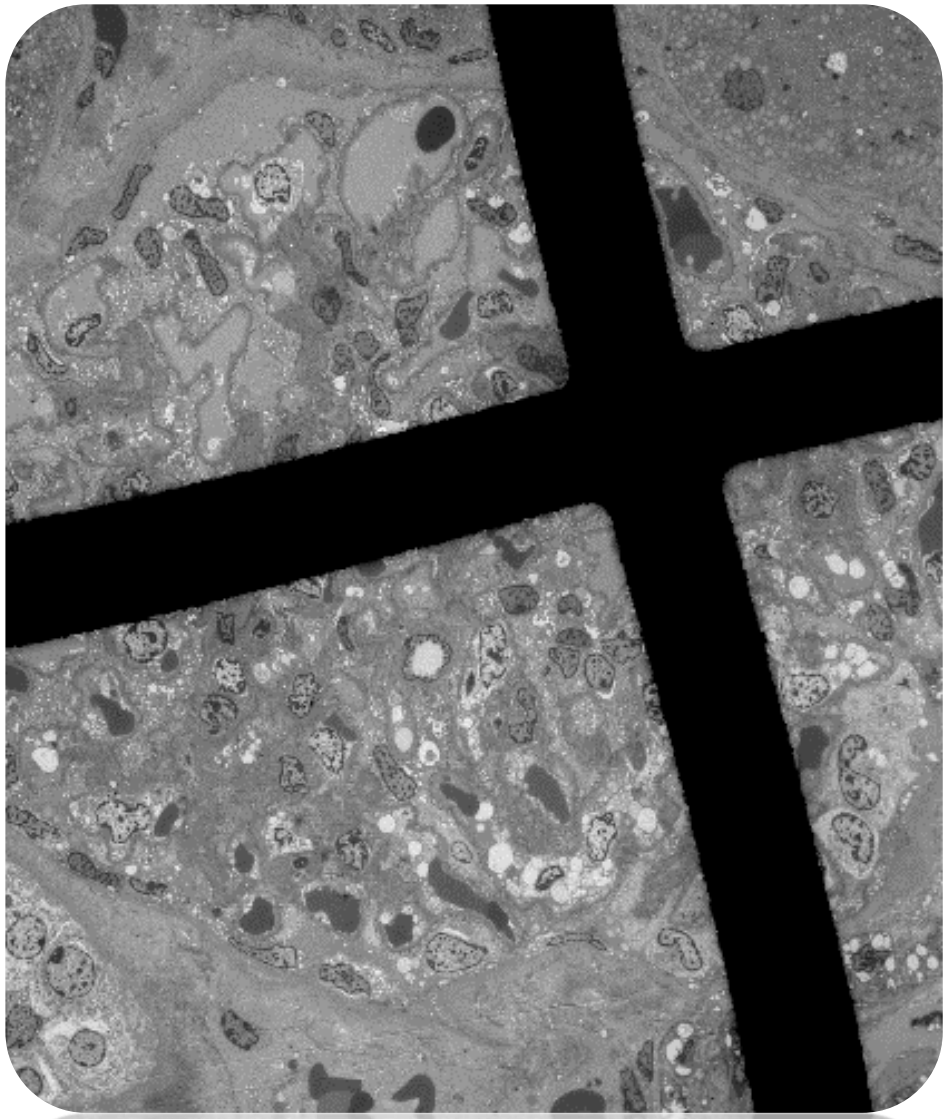


Glutaraldehyde at 5°C

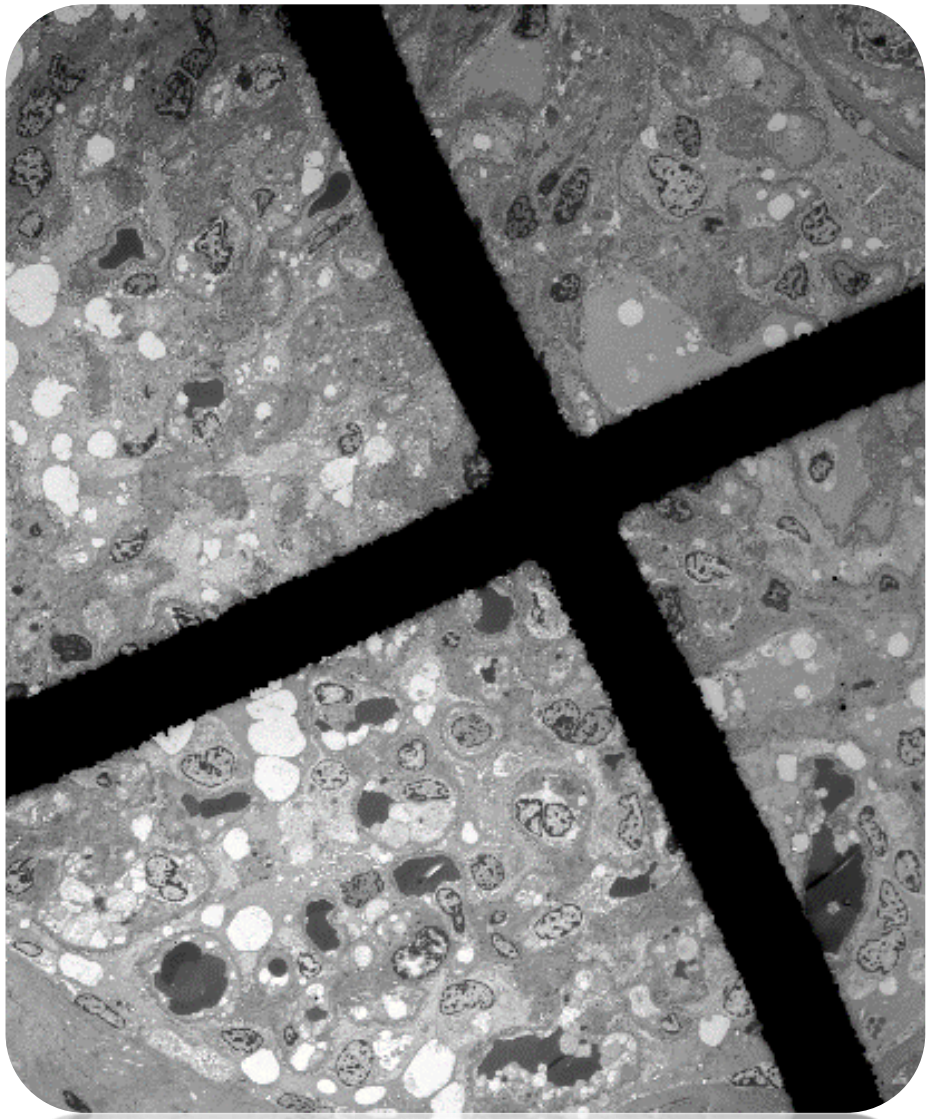


Glutaraldehyde at Room Temperature

So, a few more vacuoles, slightly more open glom – let's look a little closer….

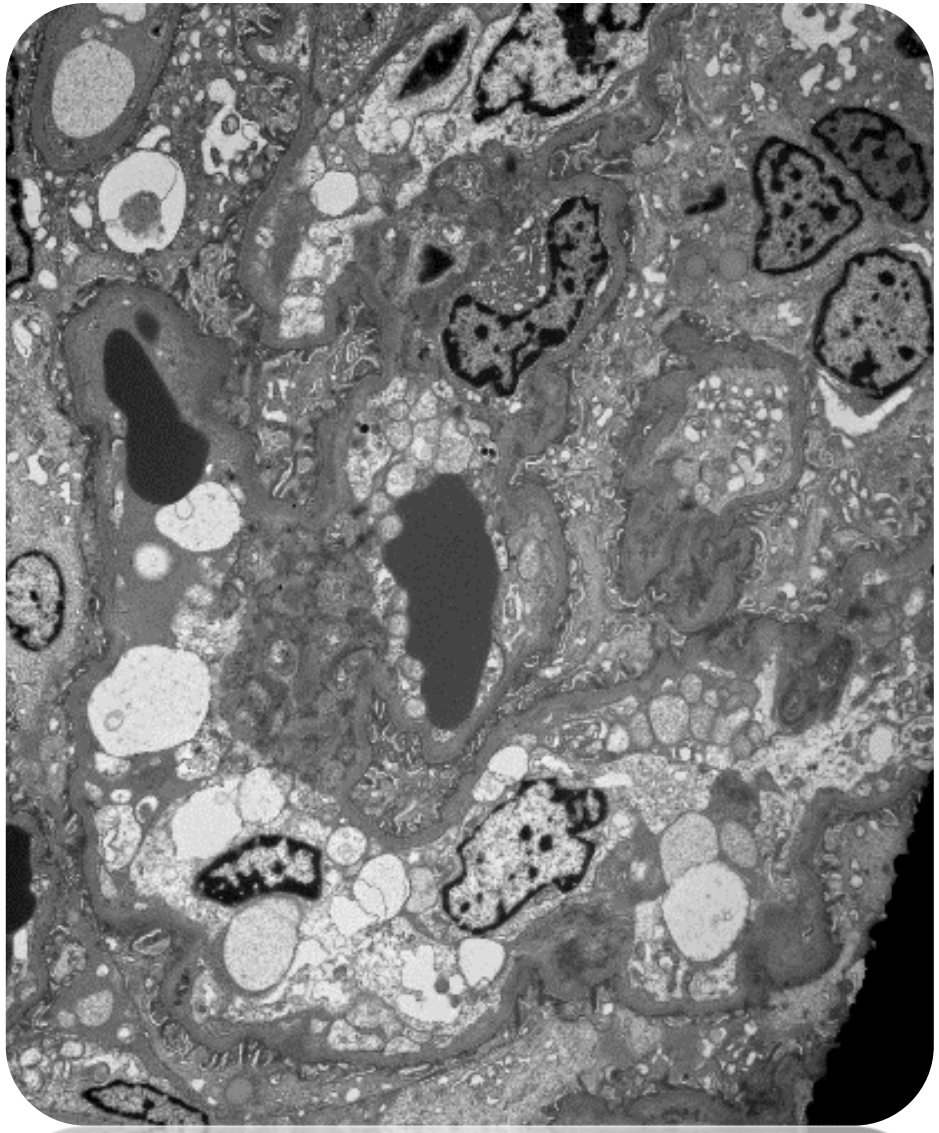


Glutaraldehyde at 5°C

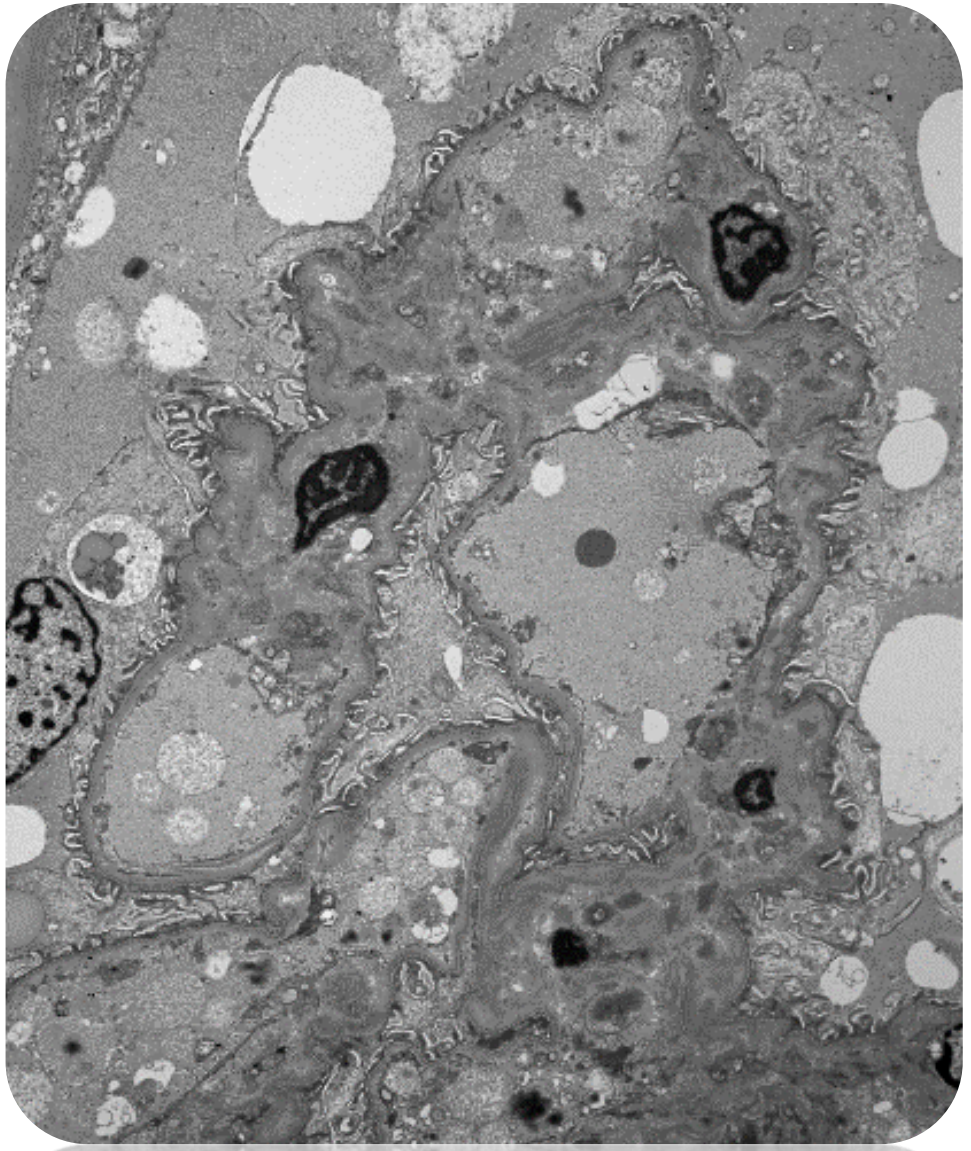


Glutaraldehyde at Room Temperature

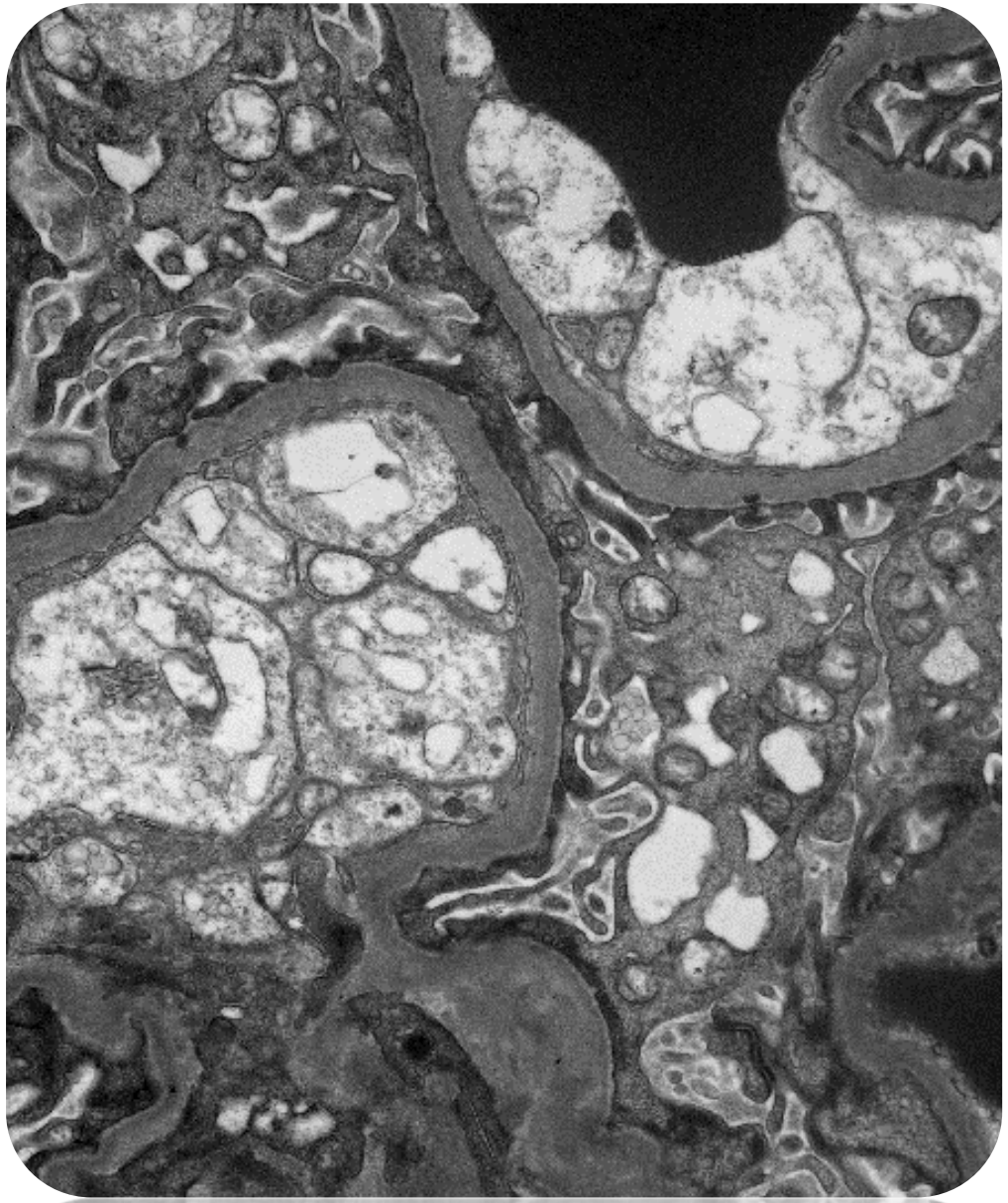
Initially, there were very little differences, but they are becoming more obvious as we move to higher powers…



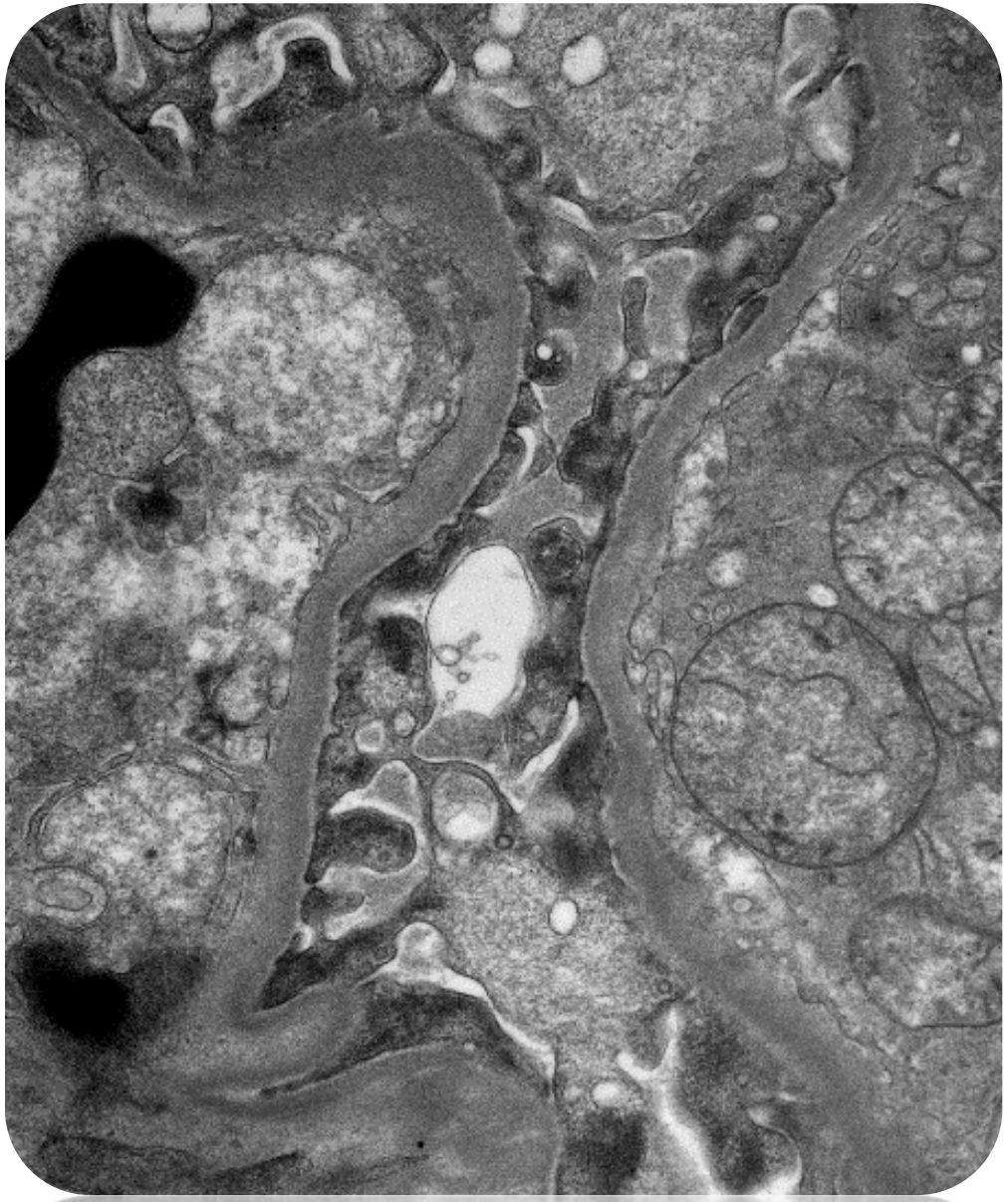
Glutaraldehyde at 5°C



Glutaraldehyde at Room Temperature



Glutaraldehyde at 5°C



Glutaraldehyde at Room Temperature

So what were the differences ?

Slightly fuzzy membranes in the room temperature sample, and not quite as crisp, but overall, these two were pretty similar and I didn't notice anything different with the samples when cutting, staining or screening the samples.

Slightly fuzzy or thickened membranes might lead us to consider diabetes, or renal ischemia or fibrosis.

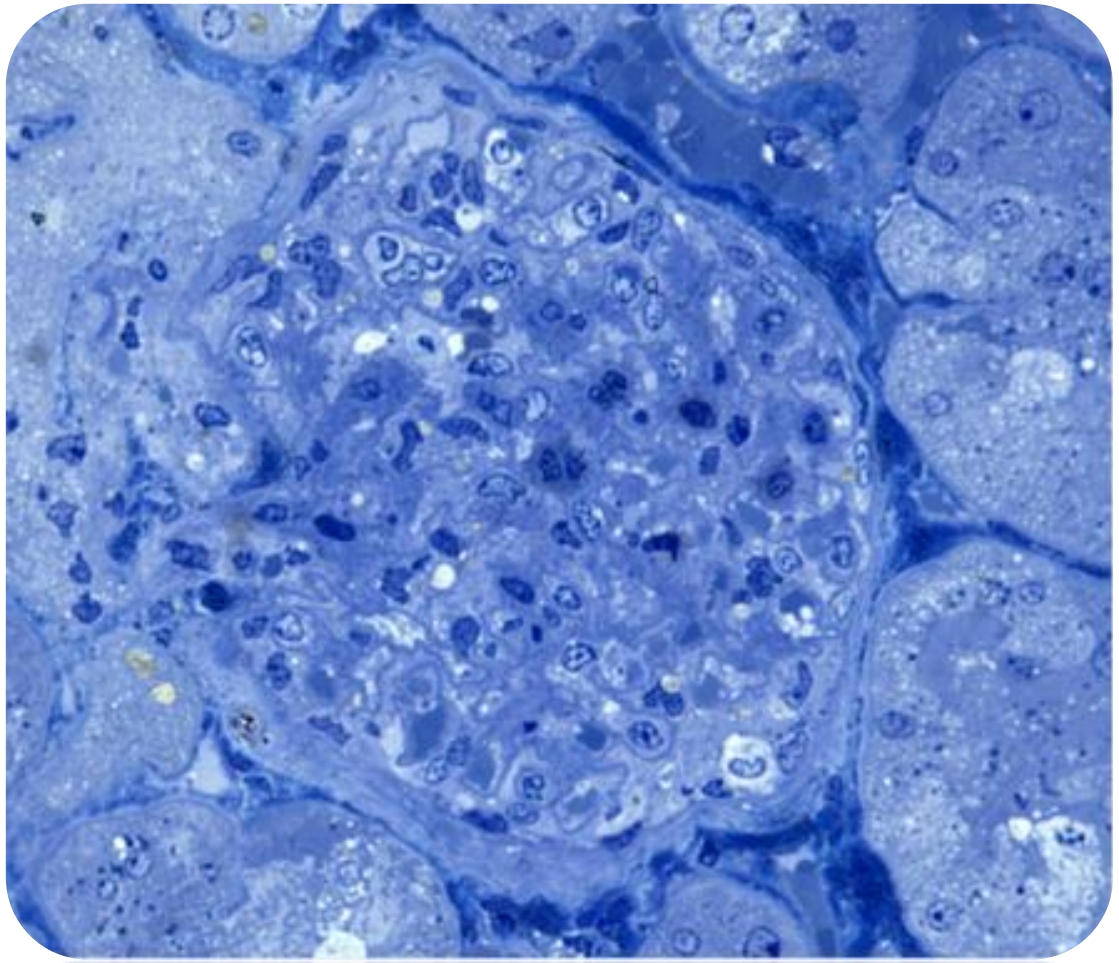
	Common conditions	Other associated findings
Thick GBM		
Negative IF		
	Diabetic nephropathy	EM-Lamina densa thick Silver stain no splitting
	Chronic thrombotic microangiopathy	Silver stain splitting of GBM
	Hereditary nephritis	Silver stain irregular EM basket weaving
Positive IF		
	Membranous nephropathy	Spike by silver stain
		Subepithlial deposits by EM
	Membranoproliferative glomerulonephritis	Silver stain GBM splitting
		Subendothlial deposits by EM
	Amyloid	Positive light chains depending on type of amyloid
	Fibrillary glomerulonephritis	Variable splitting by silver Fibril by EM
Thin GBM		
	Alport's syndrome	Silver stain irregular EM basket weaving
	Benign familial Hematuria	Thin GBM on EM

GBM: Glomerular basement membrane, EM: Electron microscopy, IF: Immunofluorescence

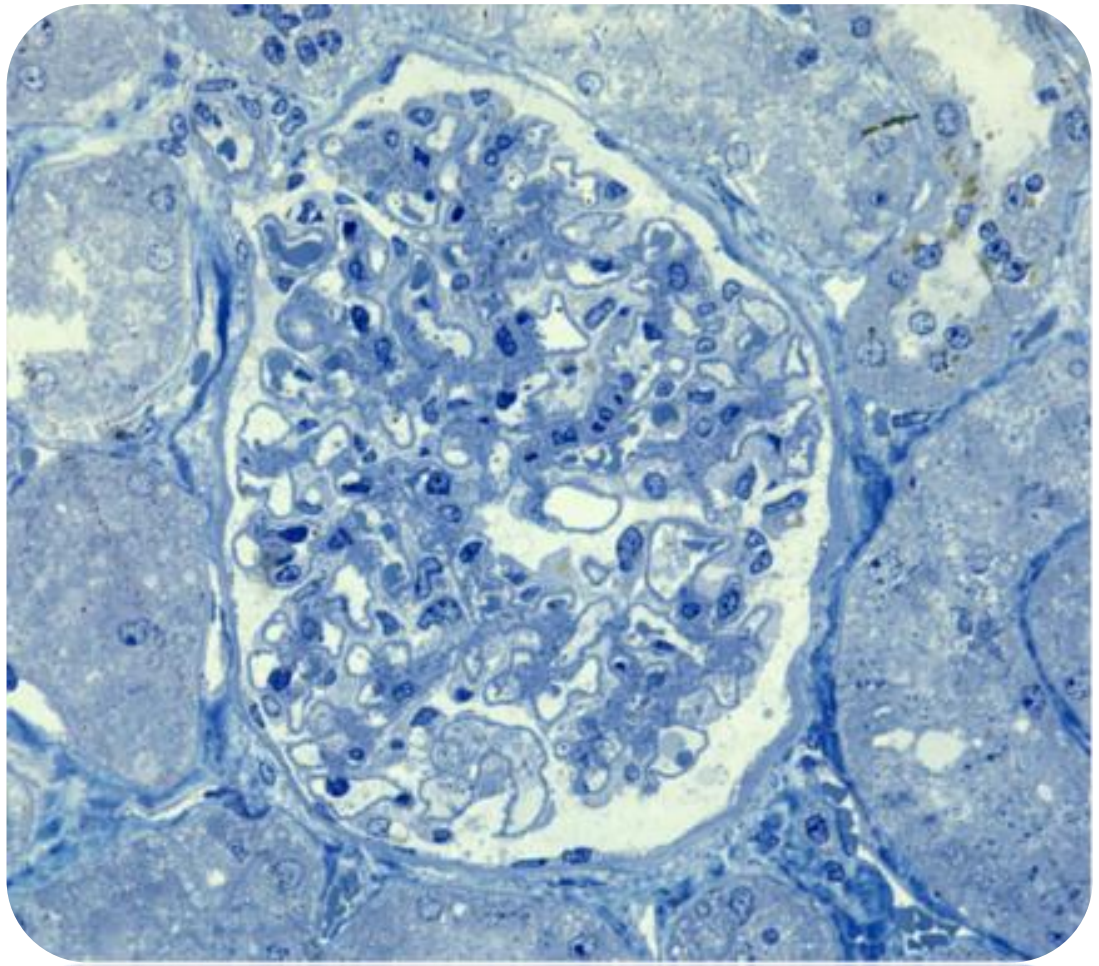
Obviously, we would never attempt renal diagnosis on an EM sample alone, and the H&E, Specials and IF are all considered by the reporting pathologist, but does this lead us down a different route if one or more of the other tests fails, or contains no glomeruli?

These differences are minor in this case, but lets continue to compare the other biopsies and look at the artefacts produced.

The next most common error in fixation is the biopsy being placed entirely into formalin whilst being transported to us:

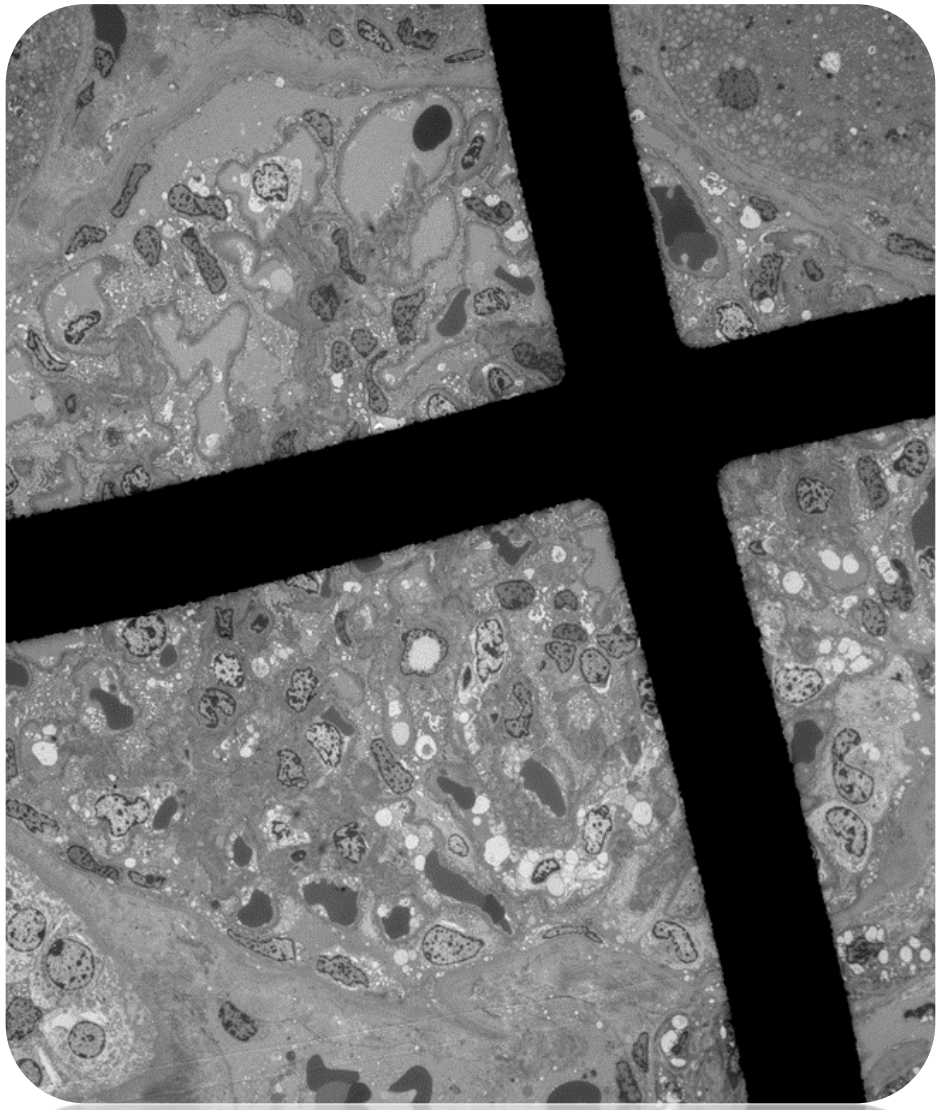


Glutaraldehyde at 5°C

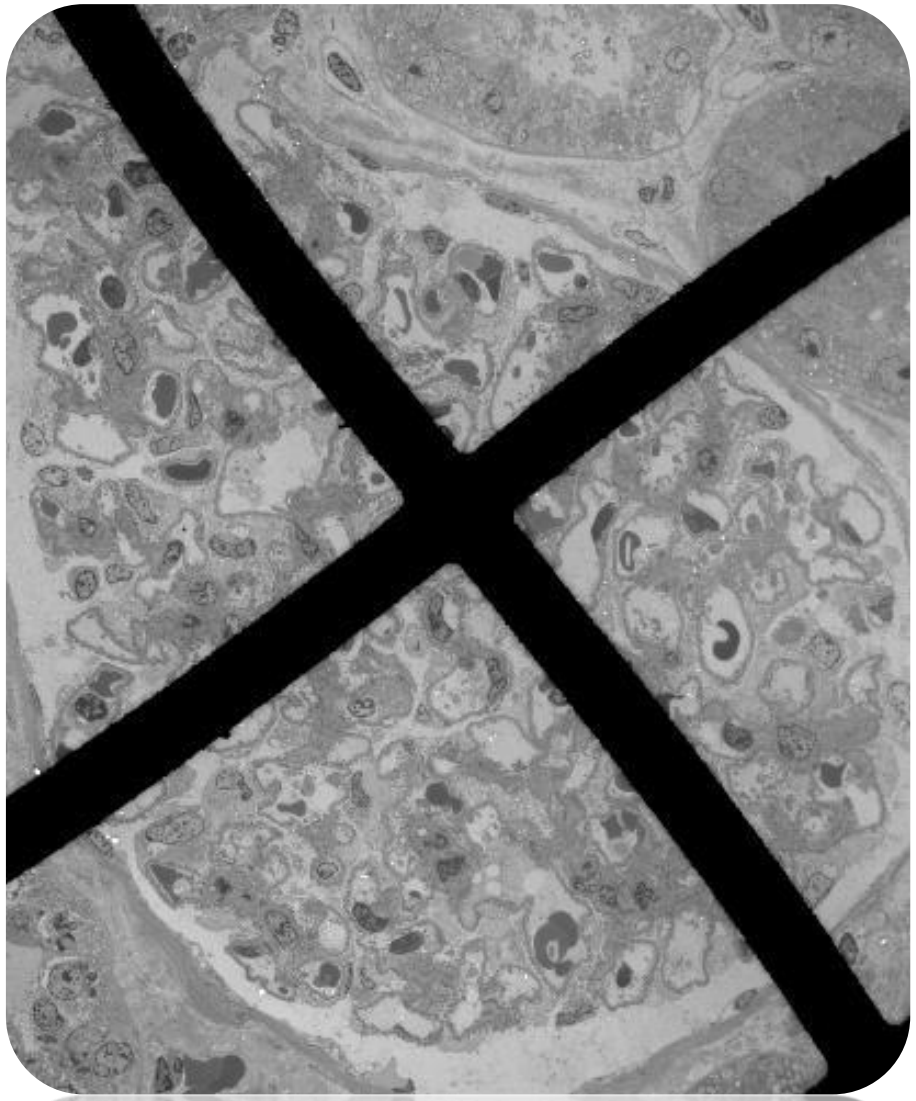


Formalin fixation

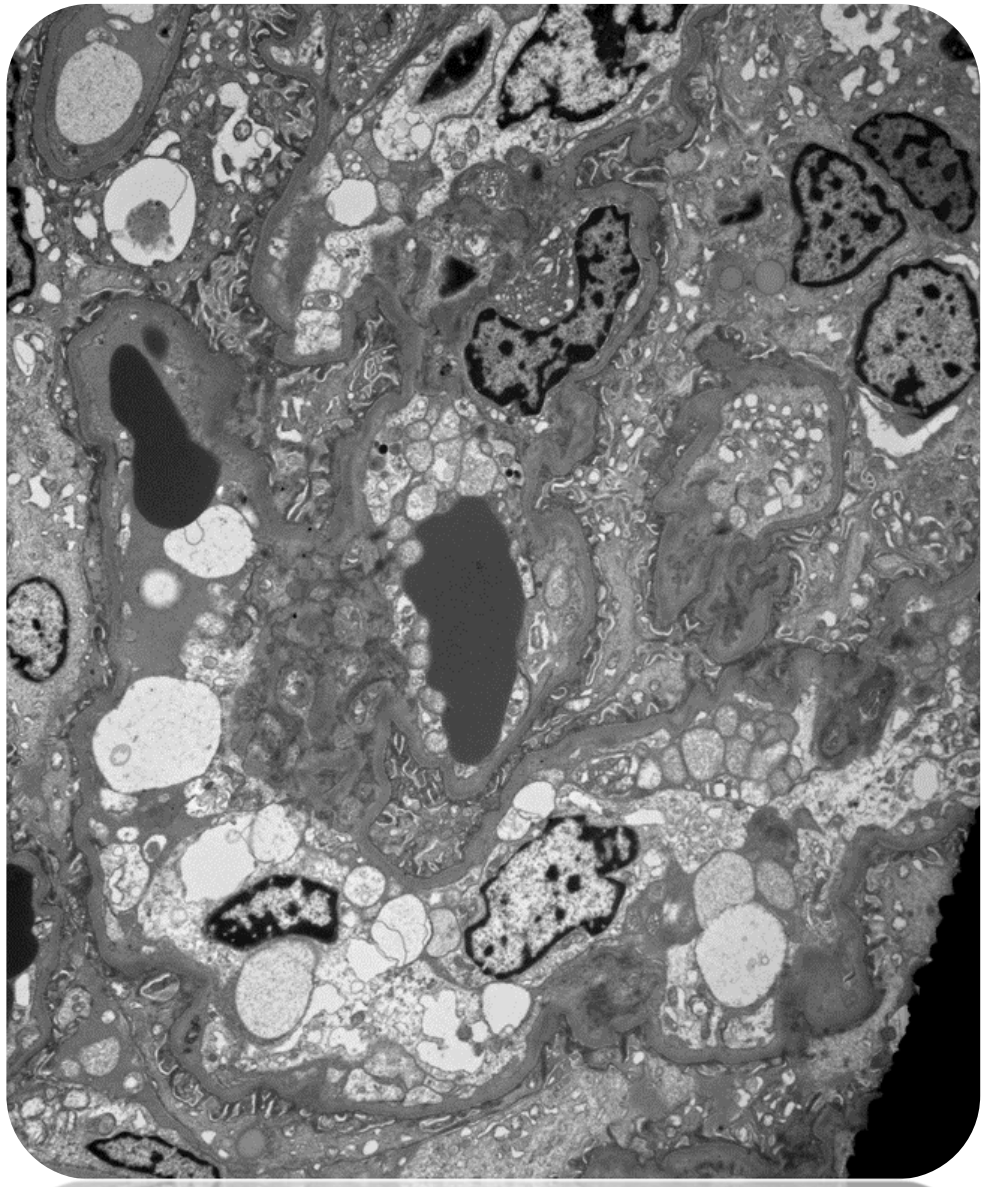
The glomerulus looks more open, with a definite space under the Bowmans capsule.



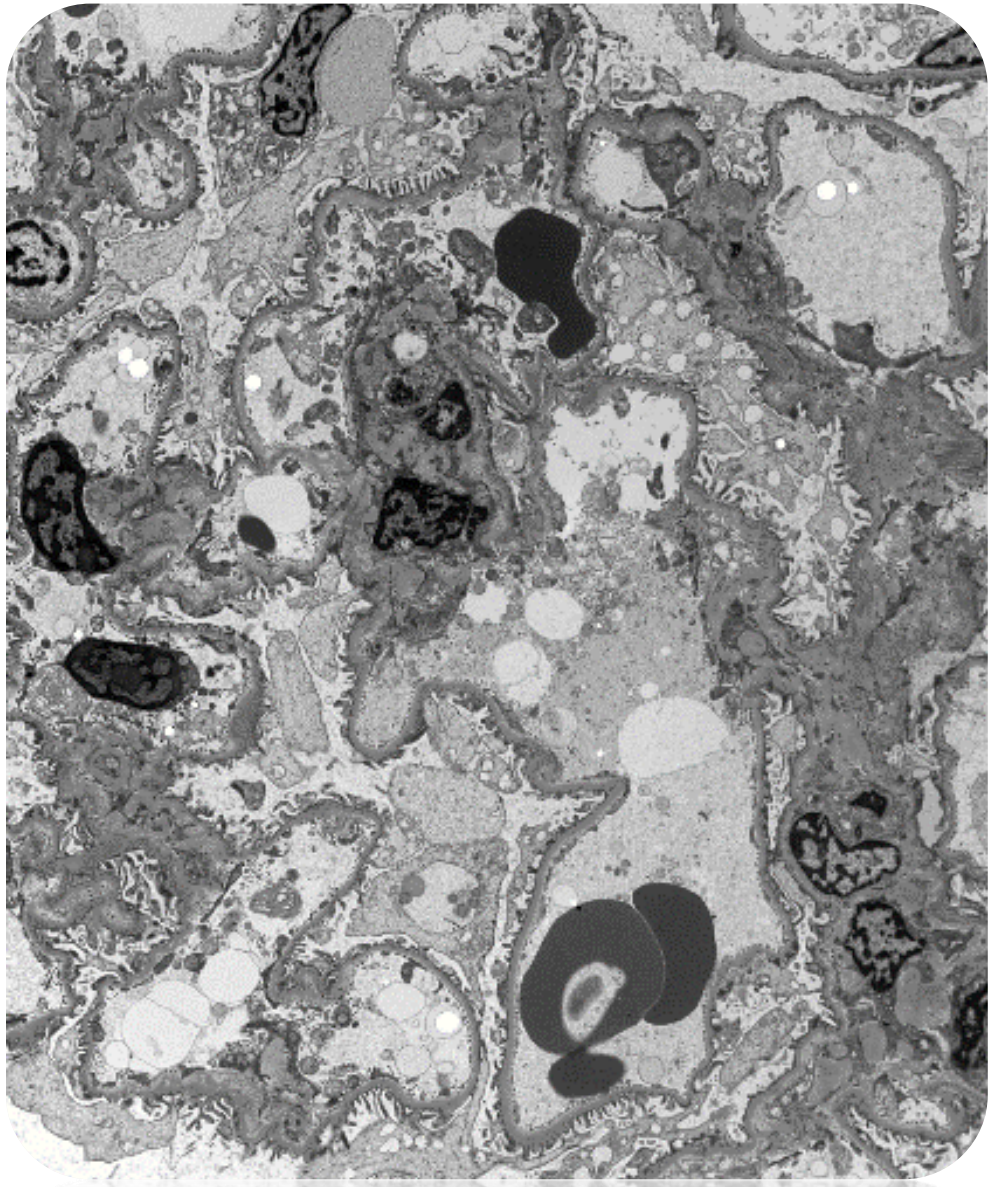
Glutaraldehyde at 5^{o}C



Formalin fixation

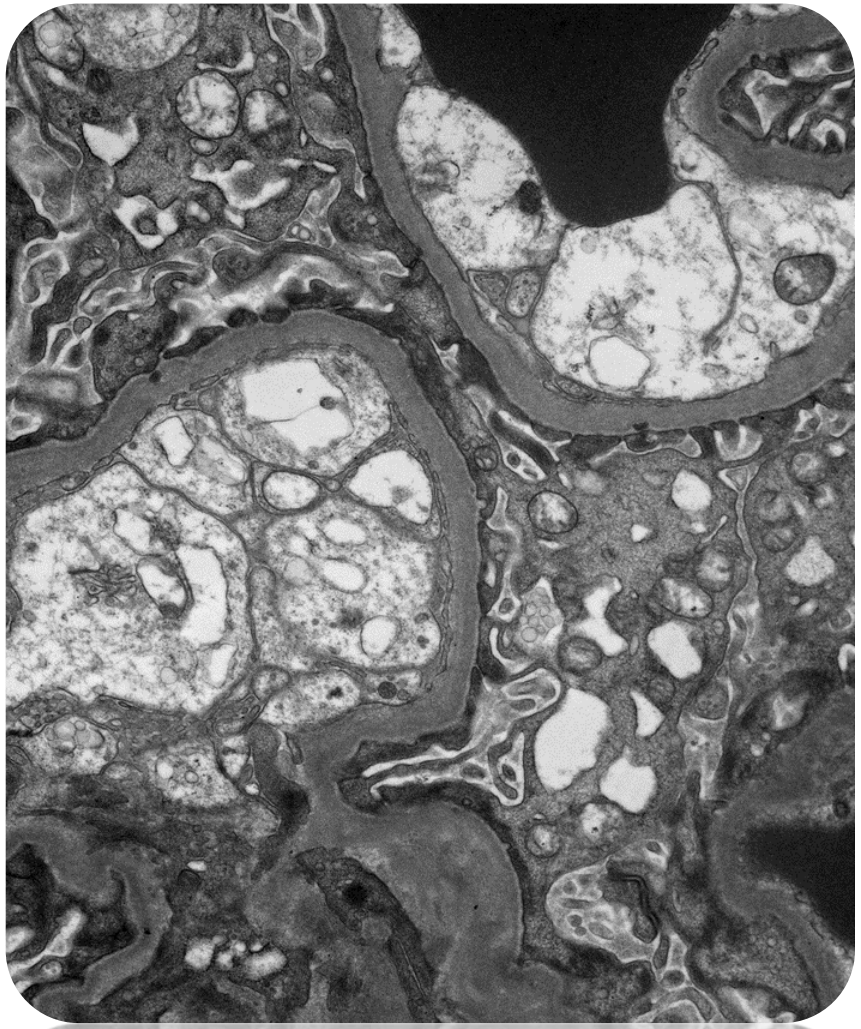


Glutaraldehyde at 5°C

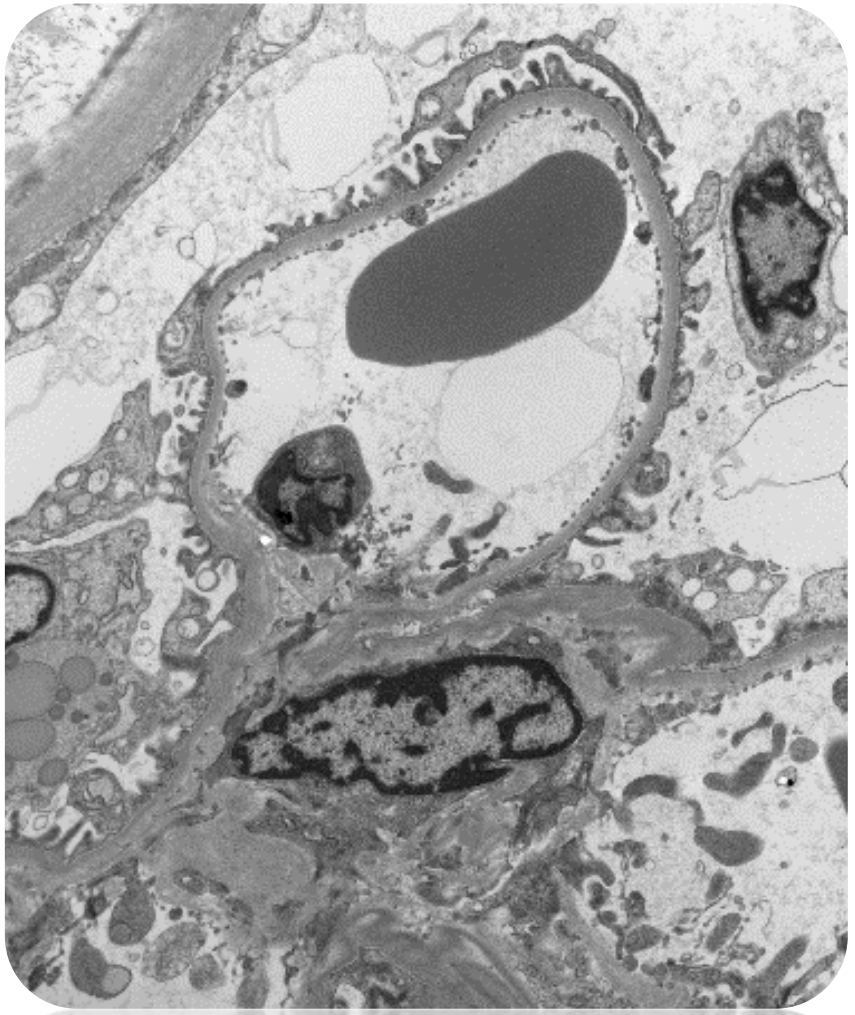


Formalin fixation

We can see the image is flatter, paler and there are areas where the podocytes have disappeared / fallen away.
Podocyte damage is now recognized as a causal factor in the progression of multiple variants of kidney disease, for example a direct correlation has been demonstrated between systemic hypertension and concomitant incidence of podocyte depletion



Glutaraldehyde at 5°C



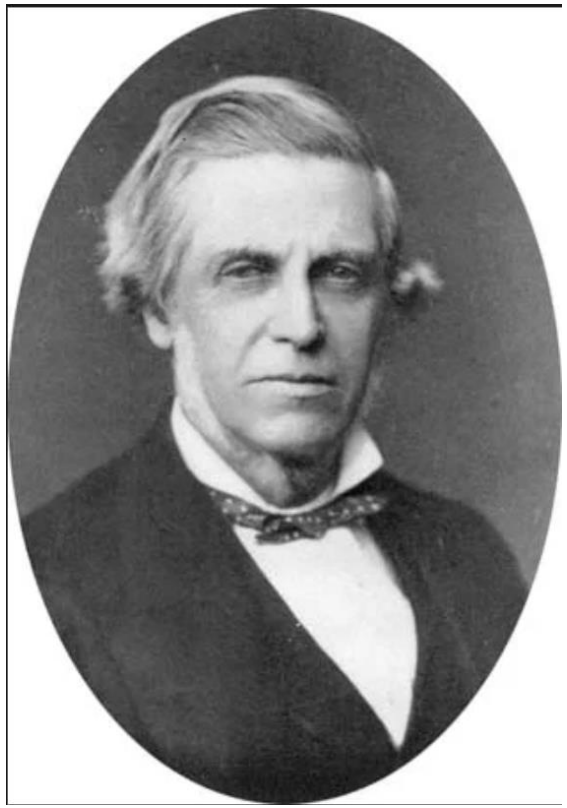
Formalin fixation

The direct correlation between podocyte depletion and the progression of glomerular disease has been well illustrated previously.
Podocyte injury causes proteinuria and eventual detachment from the glomerular basement membrane.

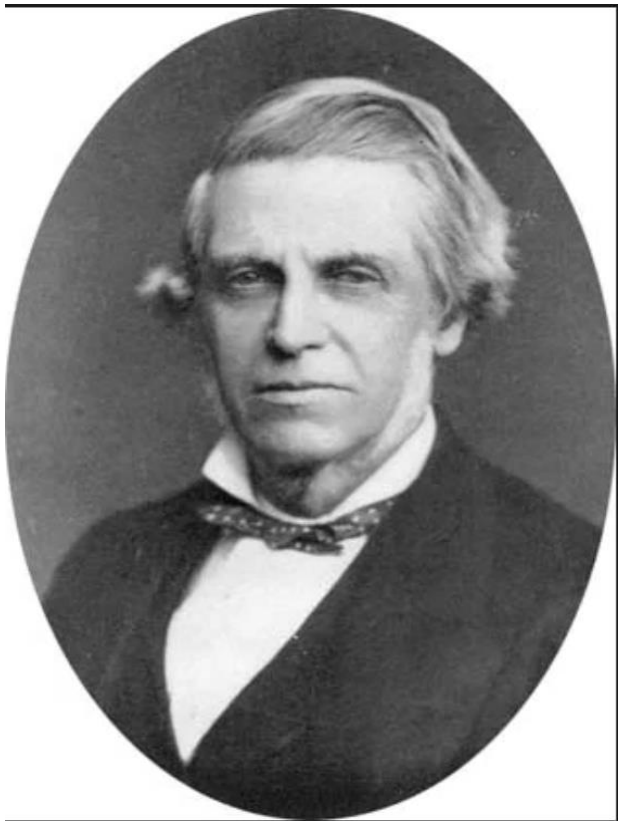
Podocyte loss is also a prerequisite for glomerulosclerosis; however, the underlying mechanism remains unclear. Podocyte death in situ on the glomerular basement membrane could result in podocyte detachment, but Podocyte detachment also leads to cell death. However, detachment of viable podocytes also occurs in many other disease processes.

The ‘false’ absence of podocytes can lead us to a conclusion of glomerular injury that is not actually present. It is important to note that these indicators are not diagnostic when viewed alone, but again can confuse us when faced with other findings

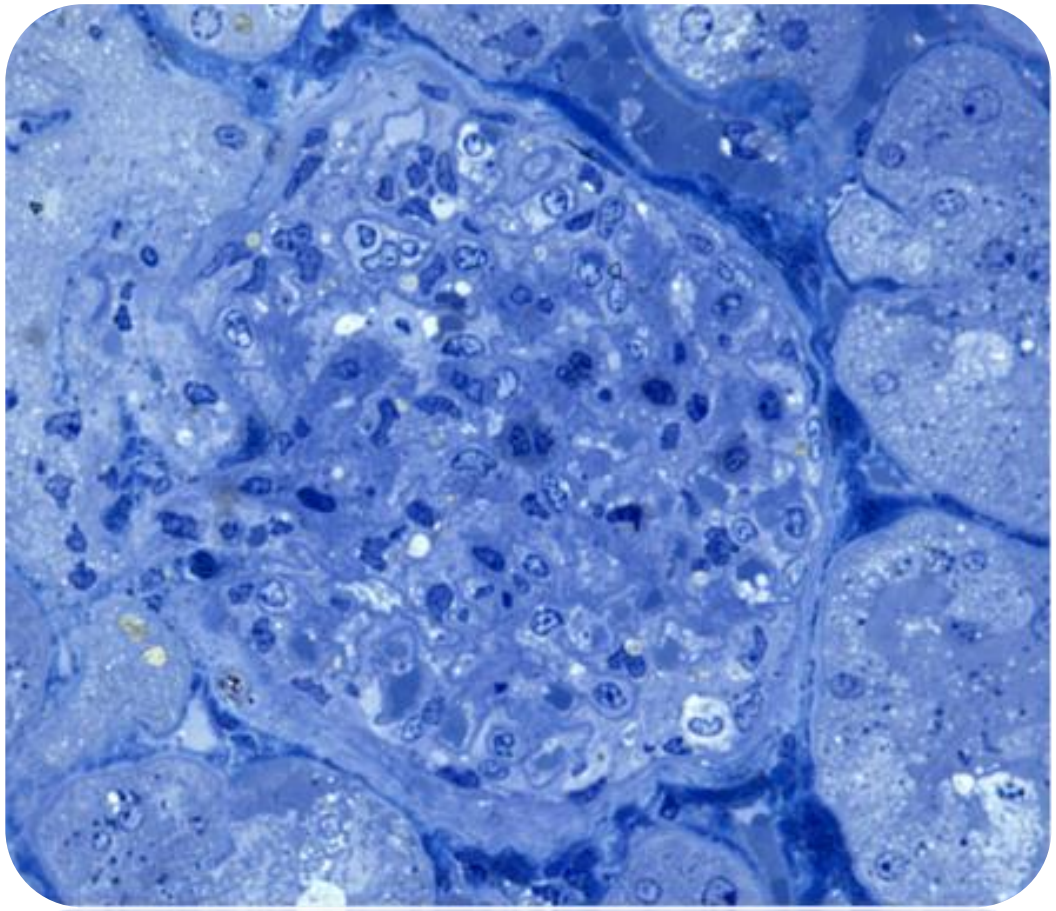
Bowman’s capsule volume is significantly influenced by age and BMI, but we are aware of the significant shrinkage that can occur during processing, so does it have a diagnostic significance?



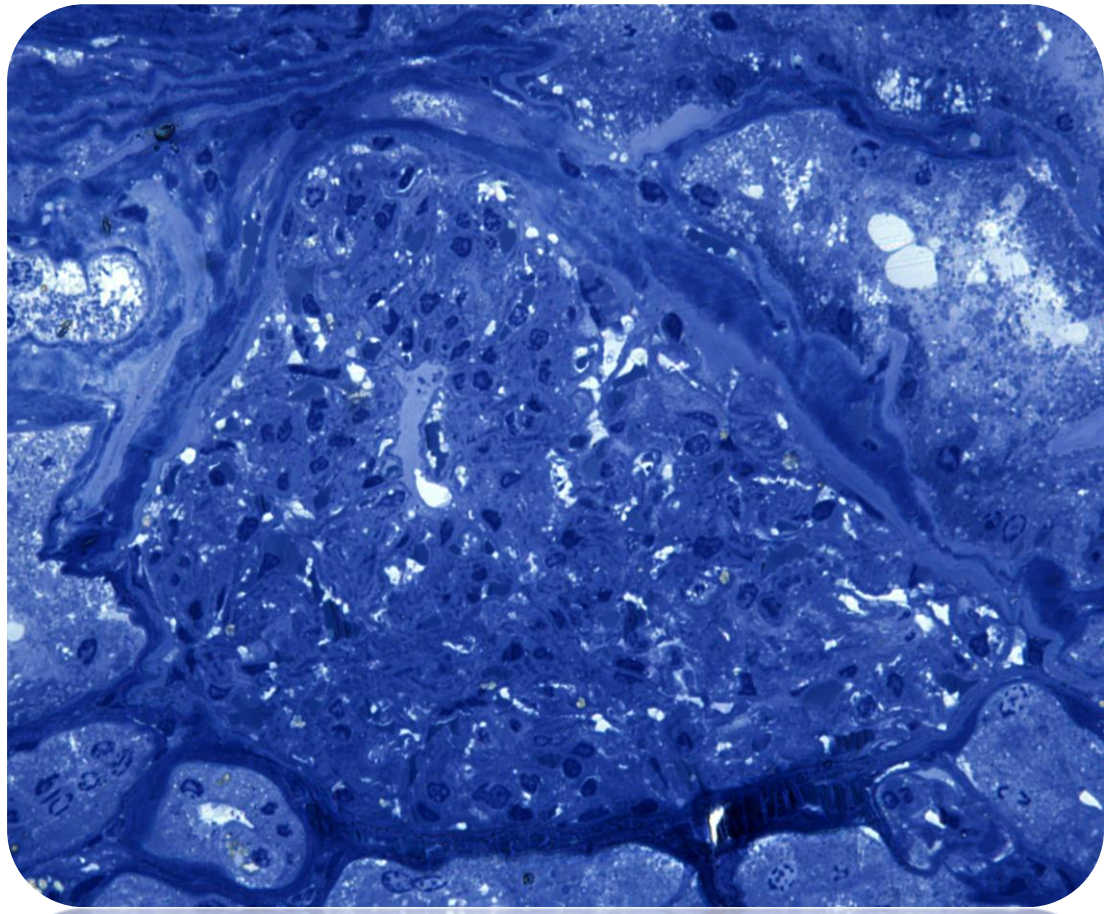
The dilation of the Bowman’s capsule around the glomeruli is an indication of Cystic kidney disease



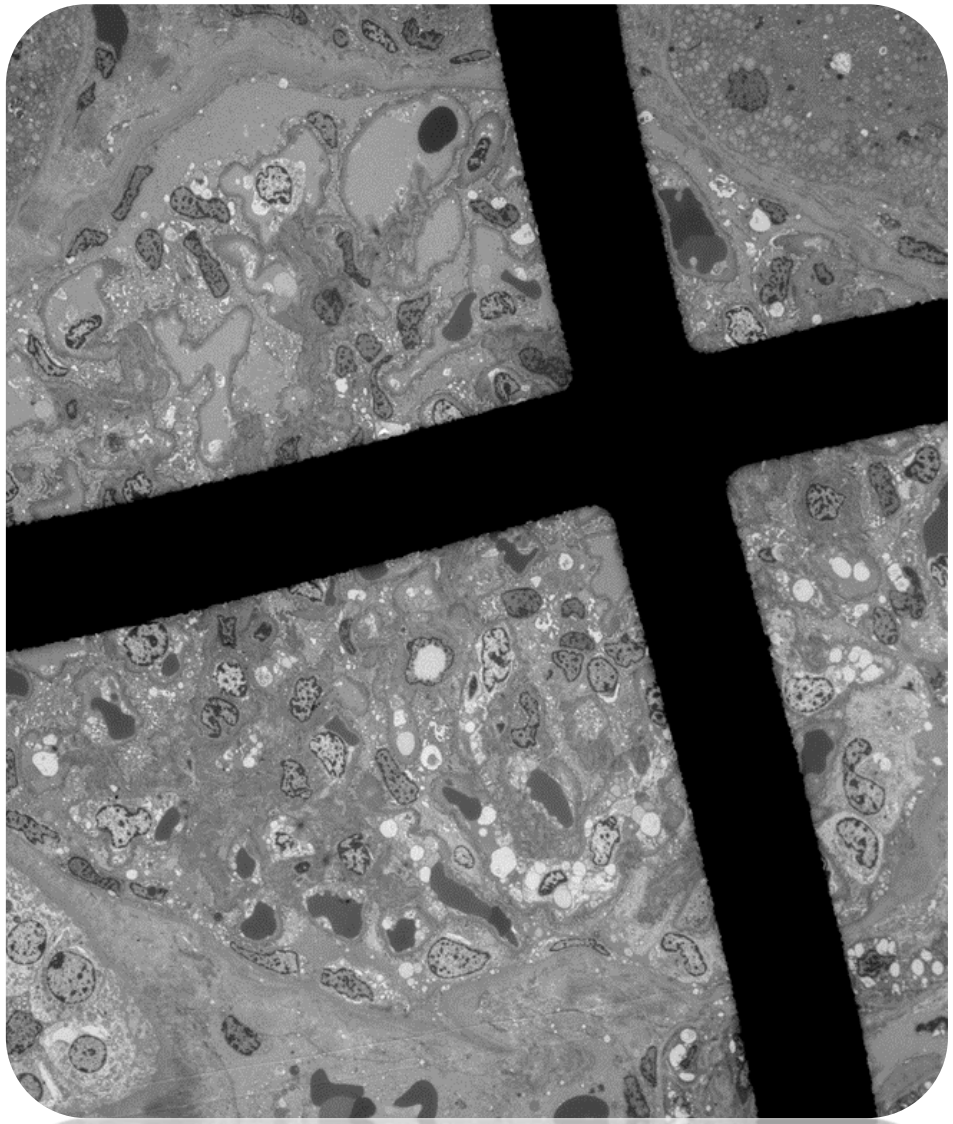
Some of our referred cases arrive to us fresh, and on dry gauze. I've calculated this to take up to 3 hours, at room temperature. We can clearly see from even the toluidine blue section that there are some major differences between what we would like as optimal handing and what we receive if the sample has dried out a little.



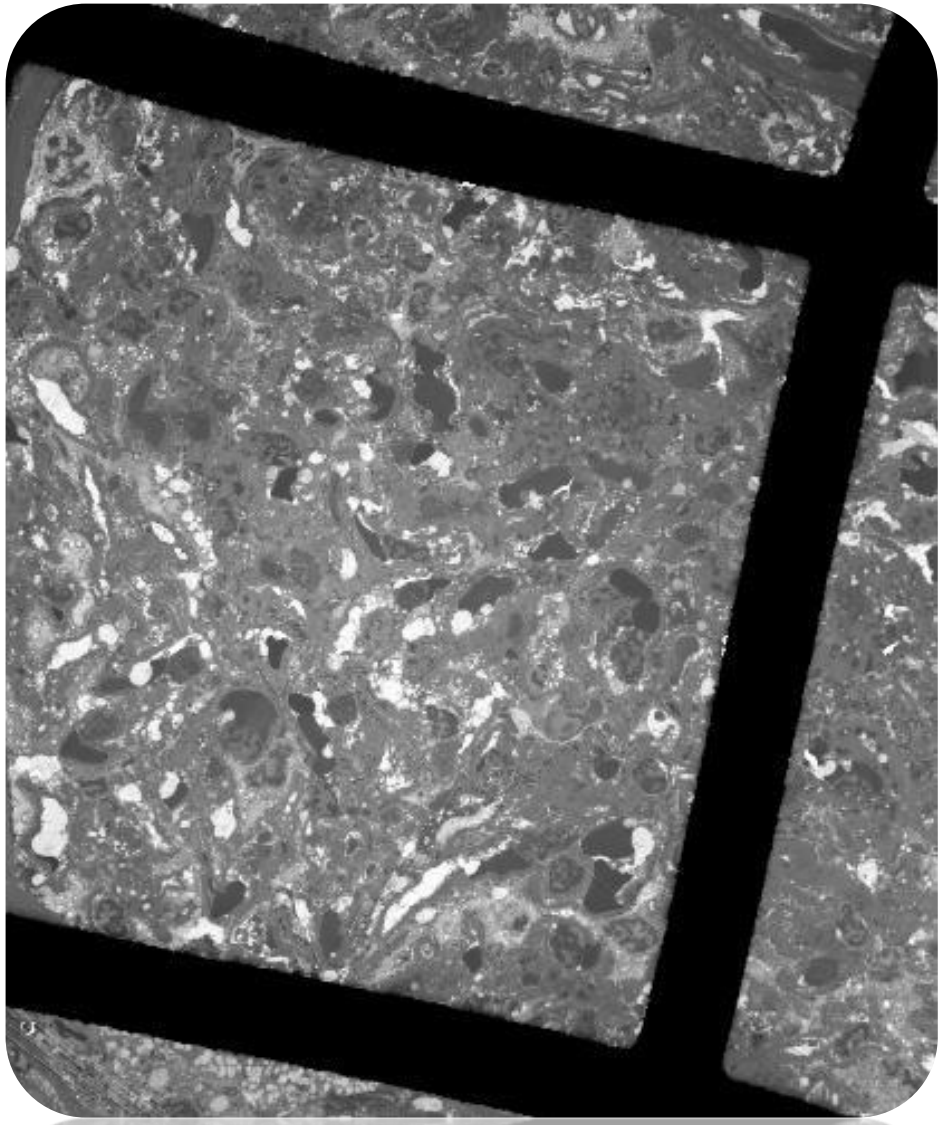
Glutaraldehyde at 5°C



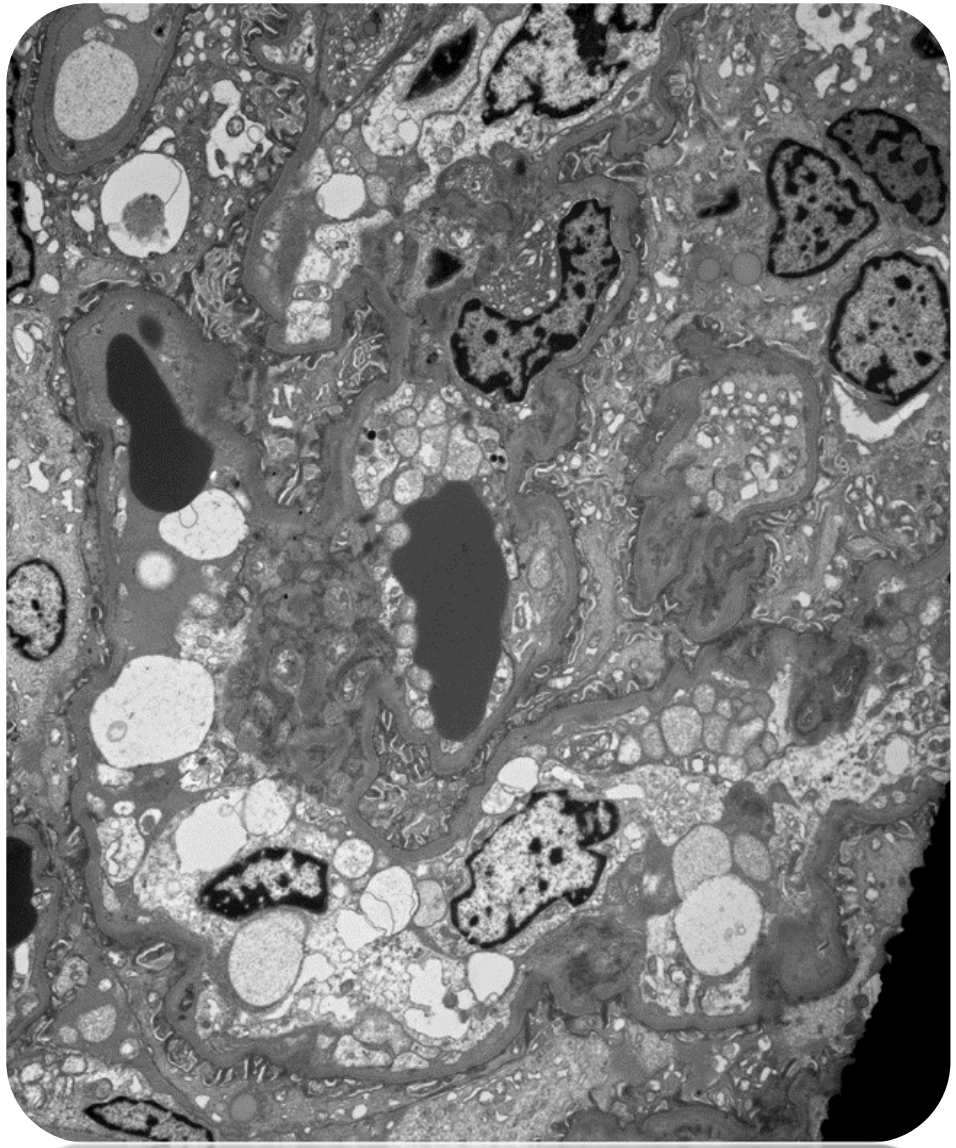
Dry



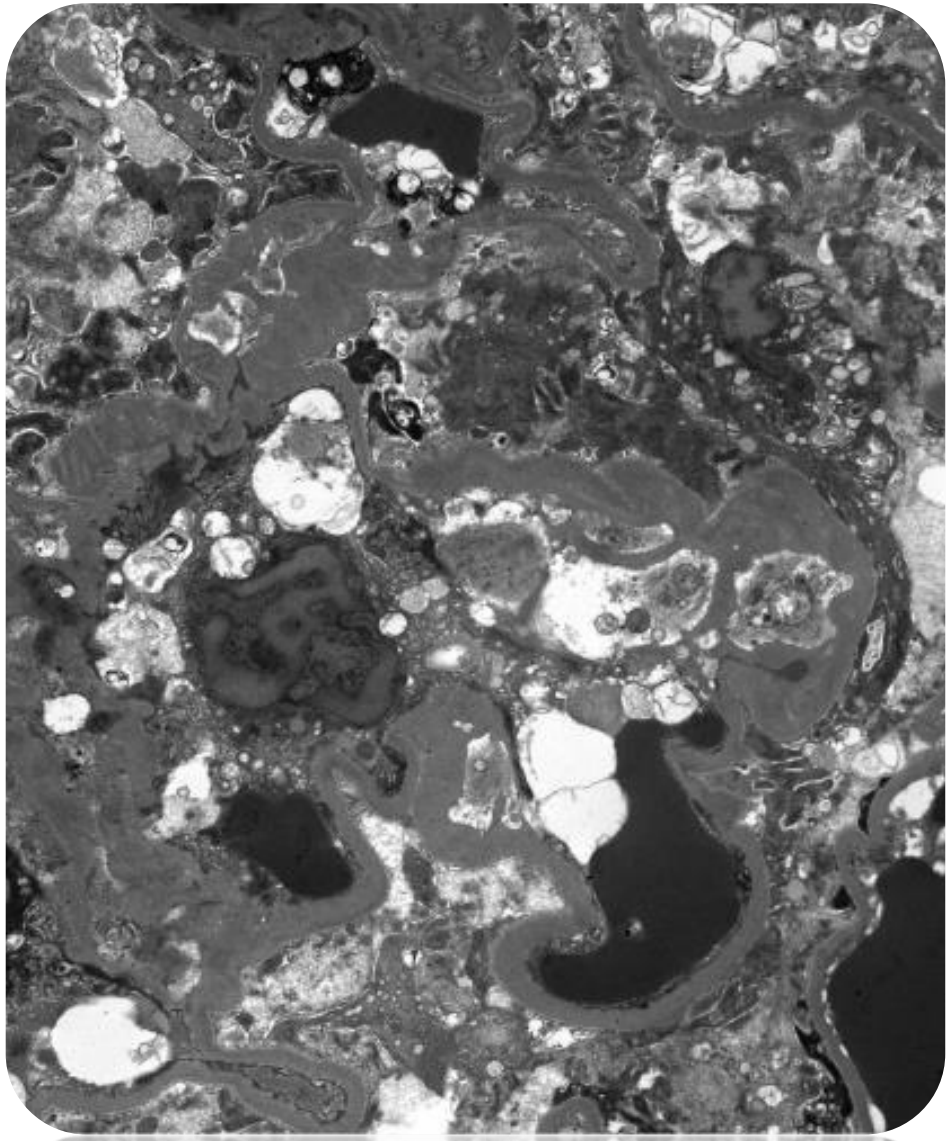
Glutaraldehyde at 5°C



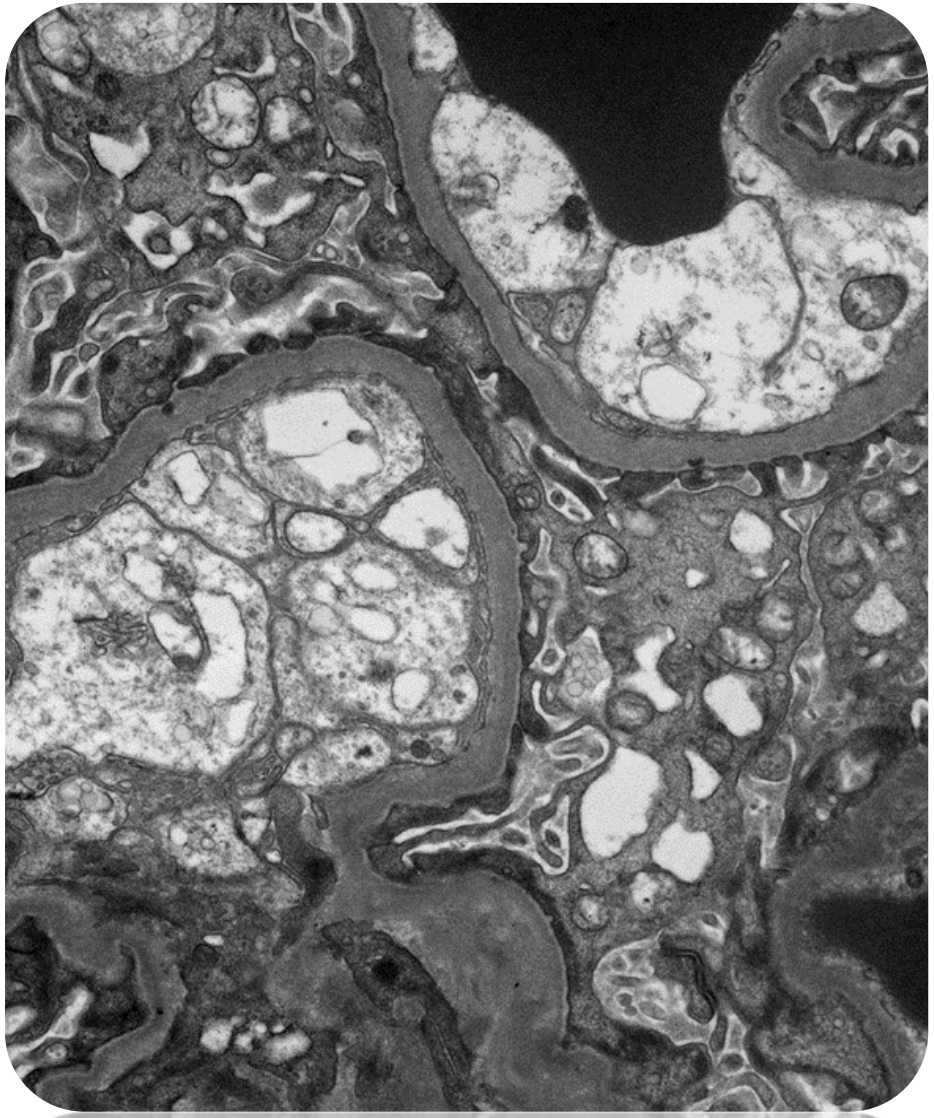
Dry



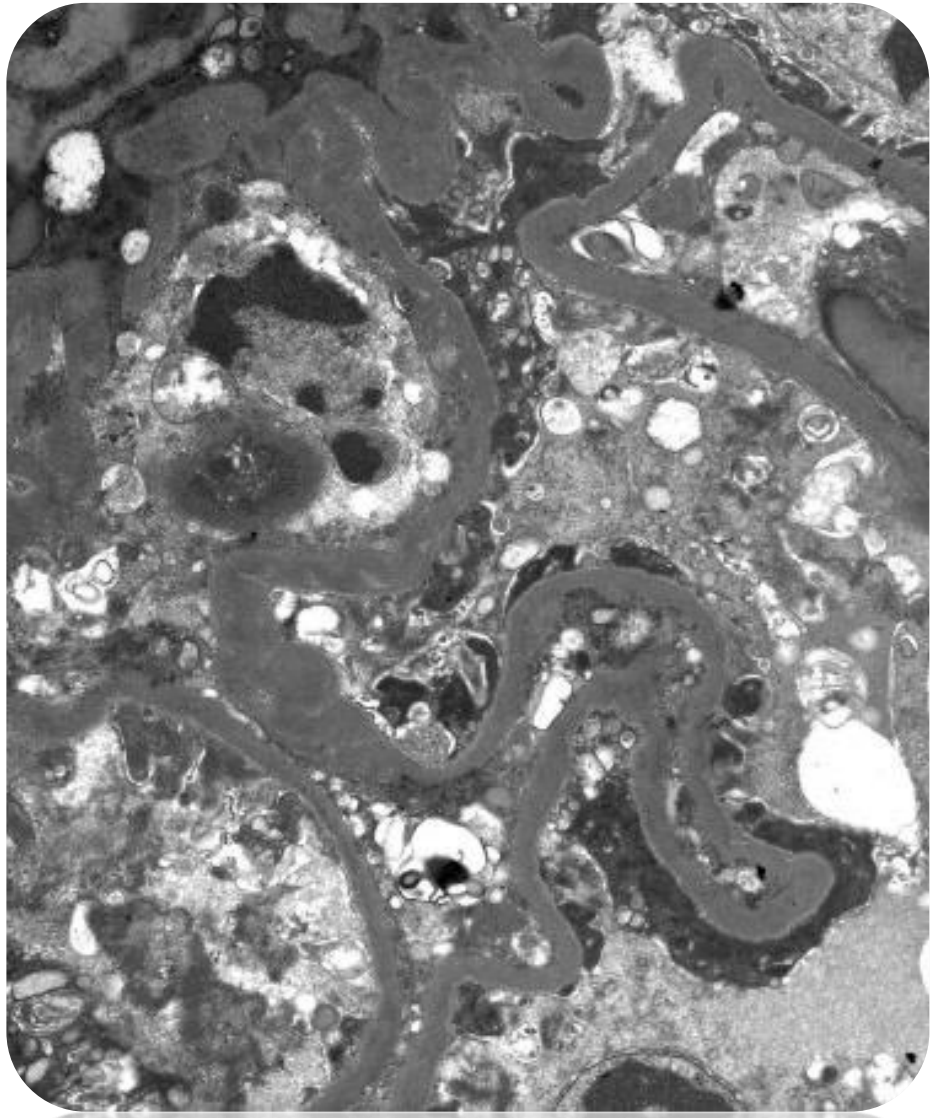
Glutaraldehyde at 5°C



Dry



Glutaraldehyde at 5°C

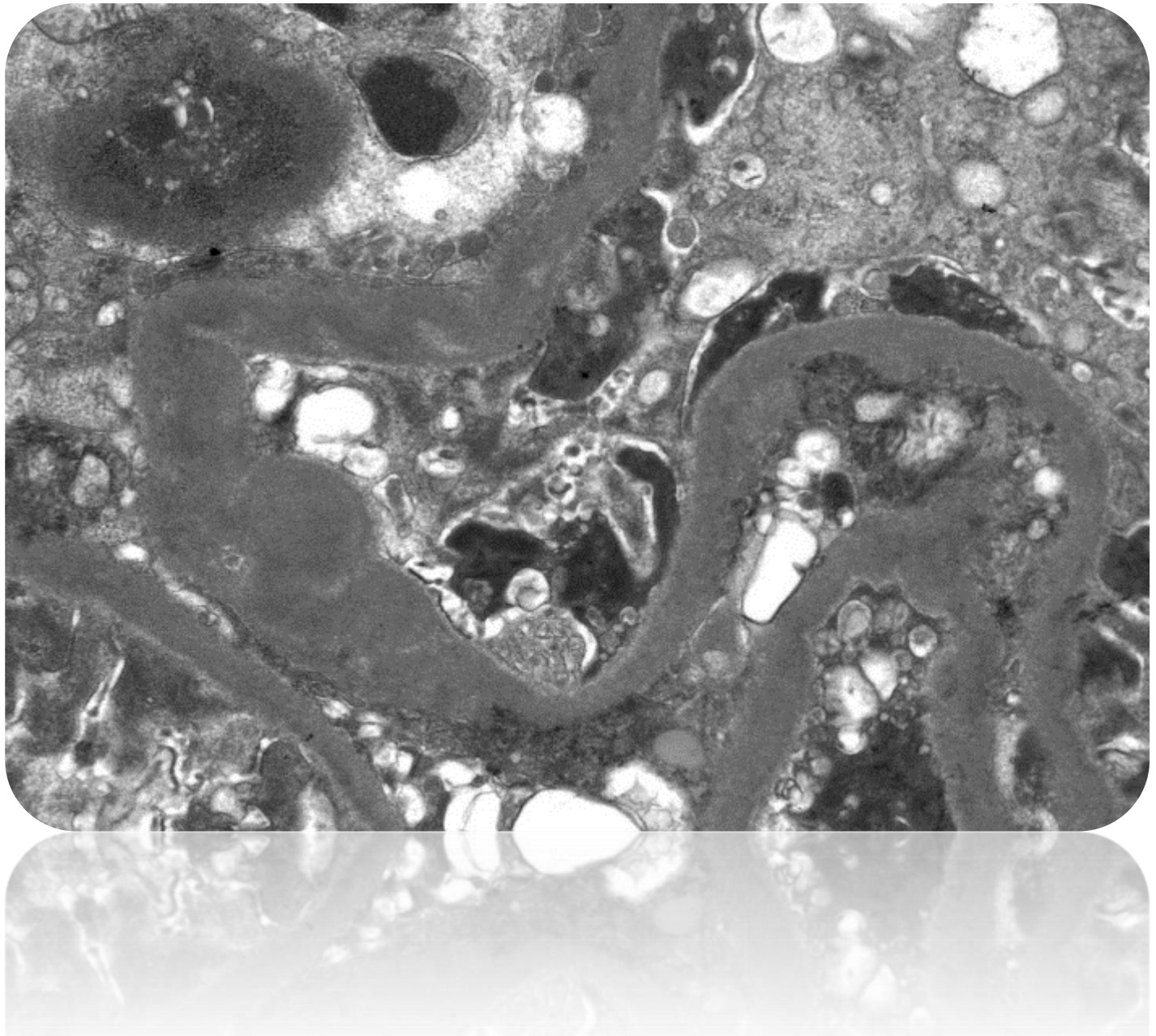


Dry

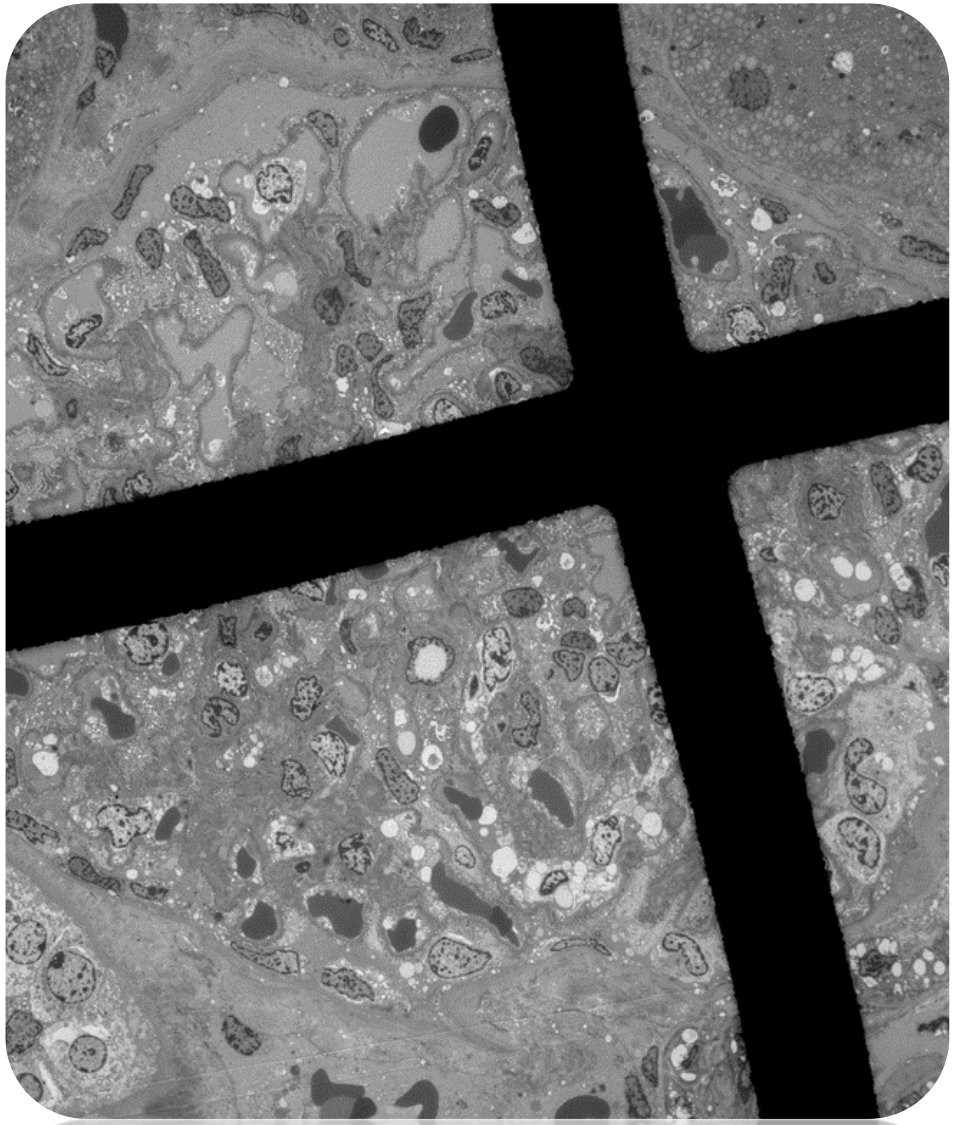
Looking more closely at this dried out biopsy, we see more wrinkling of the GBM, and uneven thickness in the diameter of the GBM – the preservation of the membrane is less than ideal, with a flat grey appearance.

Other structures such as mitochondria are very difficult to visualise, and a definite border for the endothelium is almost impossible to ascertain. Where are the fenestrae? Endothelial cell fenestrations are cytoplasmic ‘holes’, specialized for their unique role as a prerequisite for filtration across the glomerular capillary wall. Without them, the kidney could not perform its primary function of clearing low-molecular-weight waste products from the circulation.

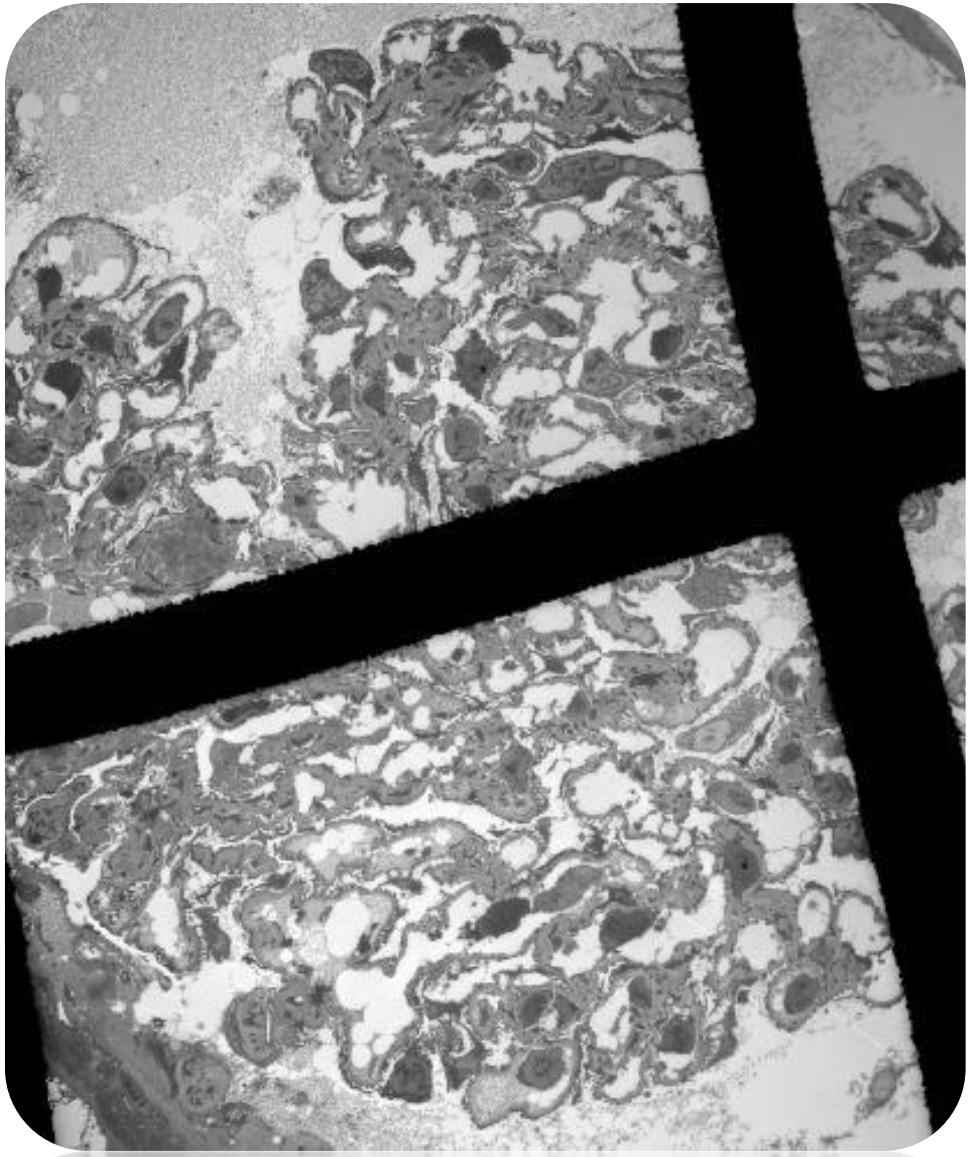
Obviously, preservation is compromised, but could we be steered towards questioning this patients other tests if the EM does not show us what we expect?



Sorry, lighting looks a bit off – due to very black epithelial and endothelial lining, which we will see in the next images

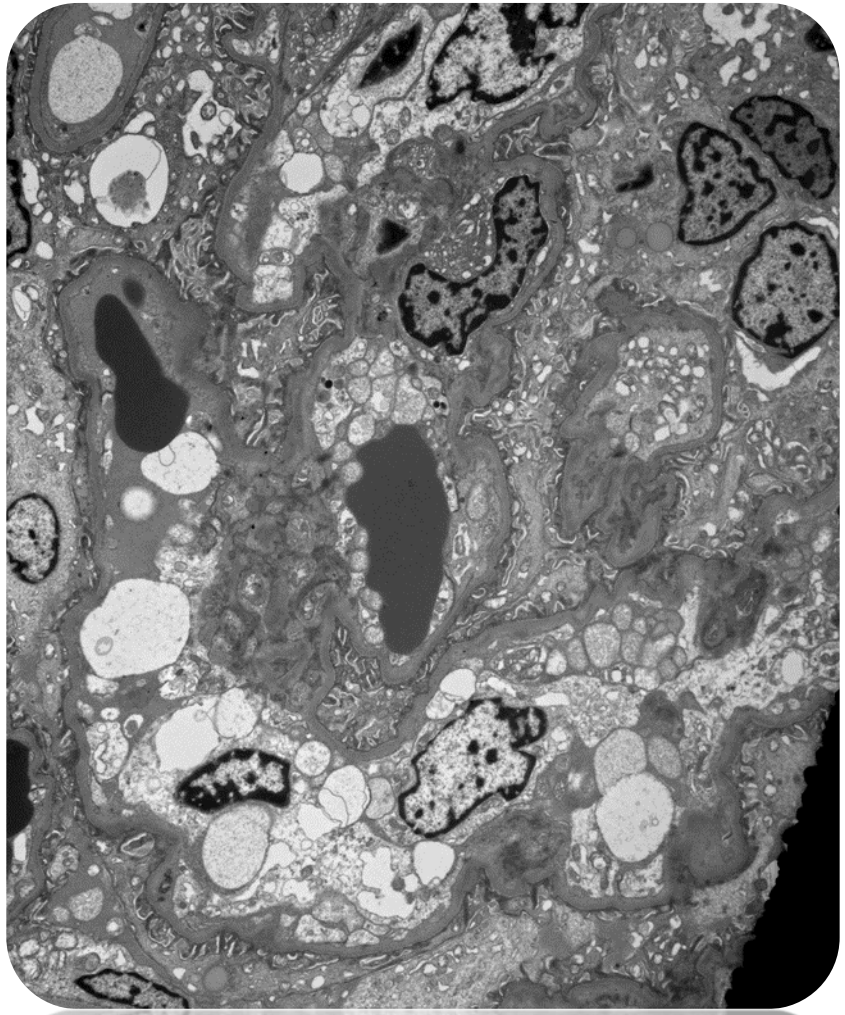


Glutaraldehyde at 5°C

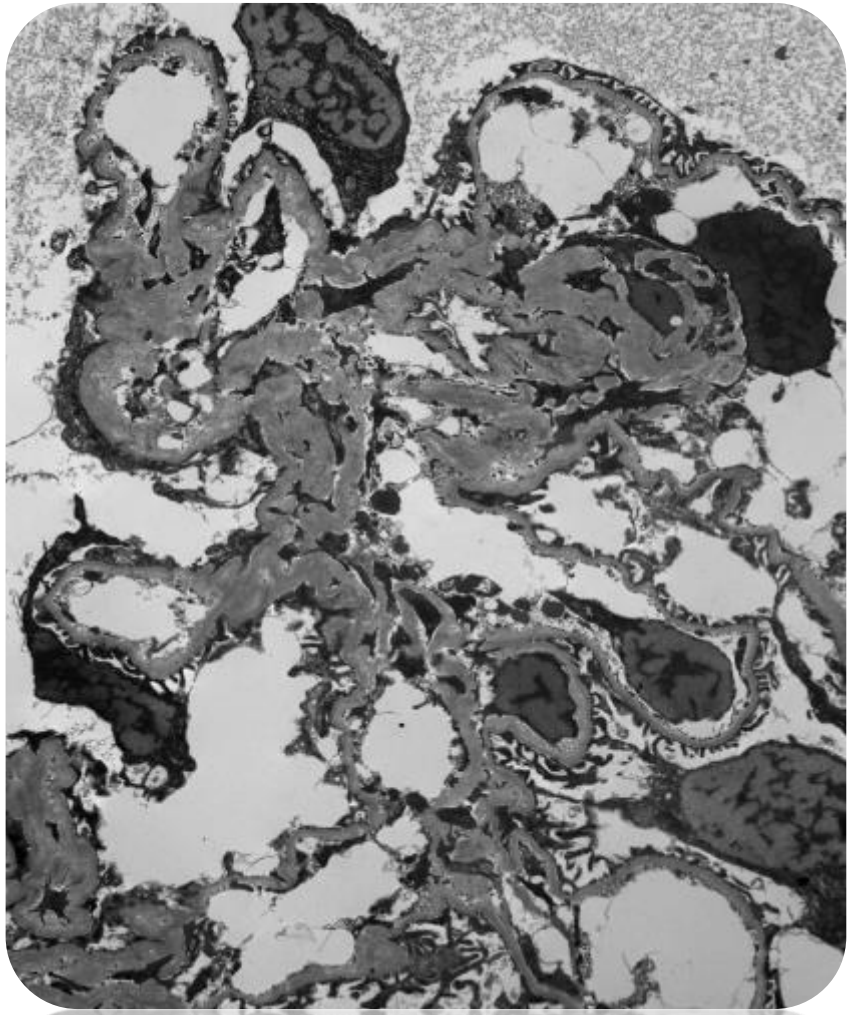


Michel's transport medium

Michel's Transport Medium is a tissue transport medium used for preservation of biopsy specimens prior to further processing, keeping the antigenic sites preserved for Immuno staining.

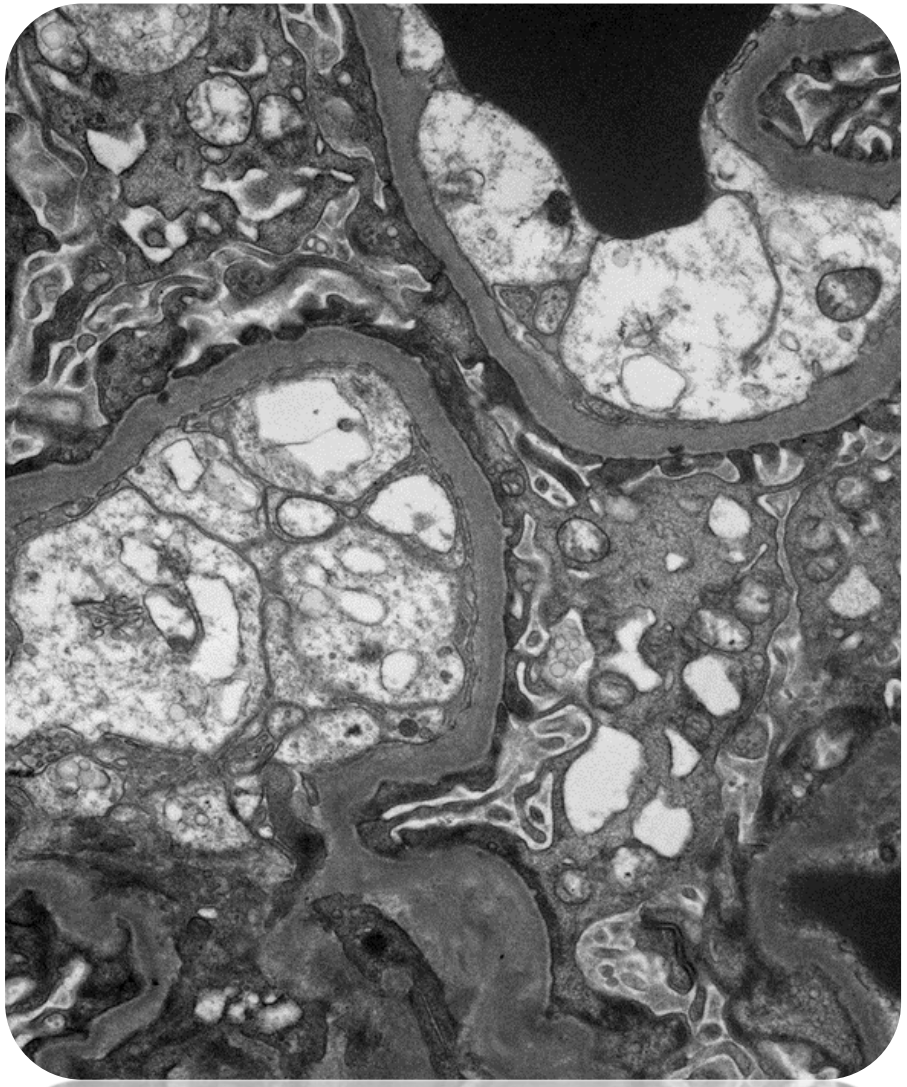


Glutaraldehyde at 5^{o}C

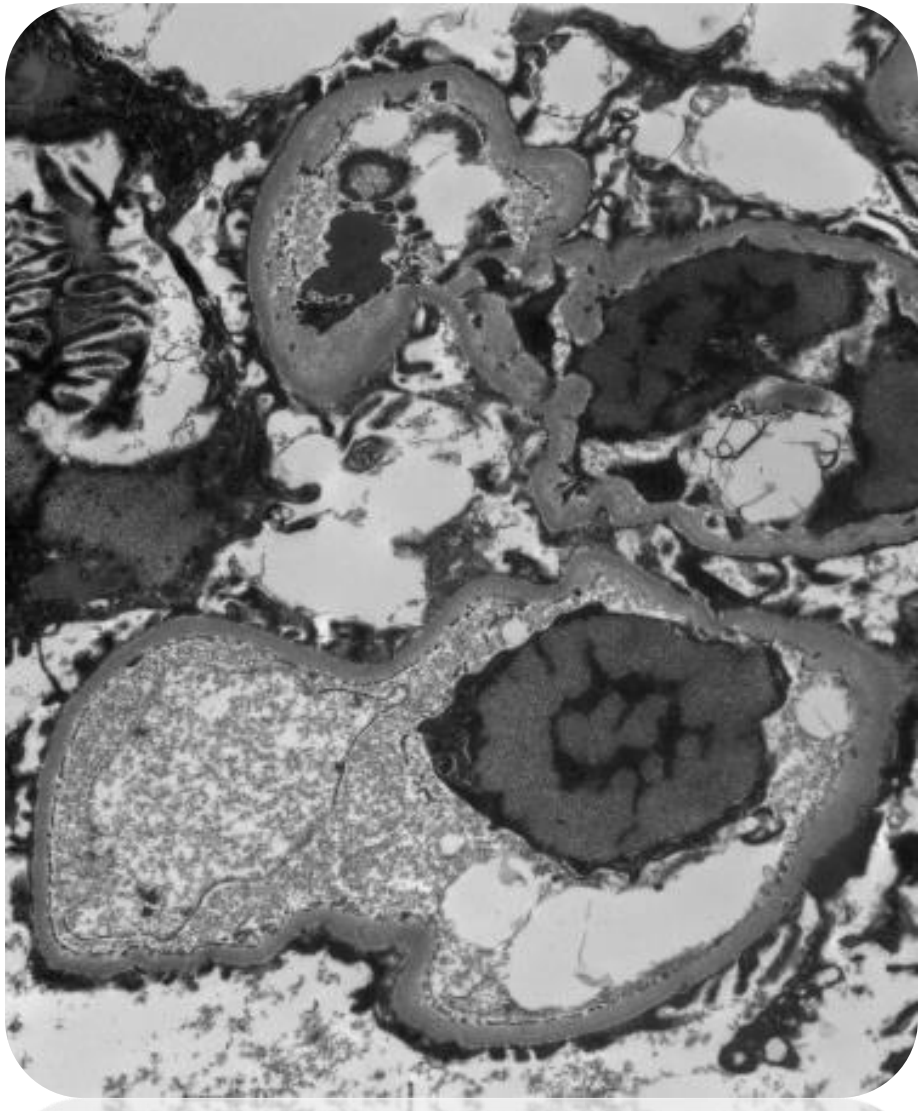


Michel’s transport medium

Oh dear



Glutaraldehyde at 5°C



Michel's transport medium

While not a fixative, specimens can be held at room temperature for five days in transport medium before processing-

This simple salt solution maintains pH, but does not kill all pathogens

Specimens received in transport medium should be washed prior to further studies, but does this washing make more of a difference or is it worse without?

Does the medium react adversely with Glutaraldehyde?

So what can we see and what's missing?

There is a total lack of endothelial cell cytoplasm and endothelial call margins.

There are very few attached podocytes, and foot processes appear much darker than with routine fixation.

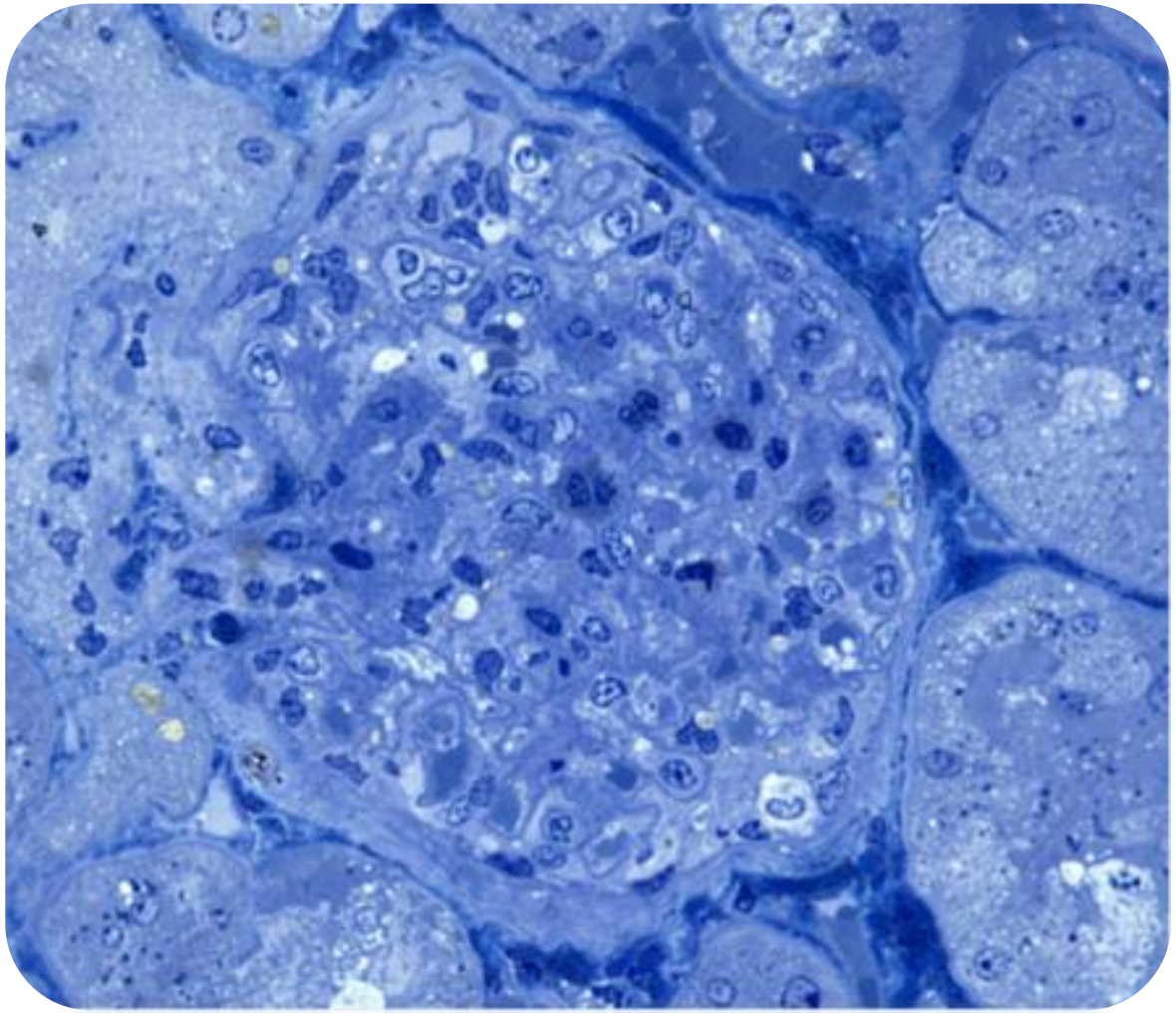
The membranes appear fuzzy on higher power images and Endothelial cell cytoplasm is distorted .

Is there endothelial lucency or not? There is certainly some effect on the endothelial side of the membrane

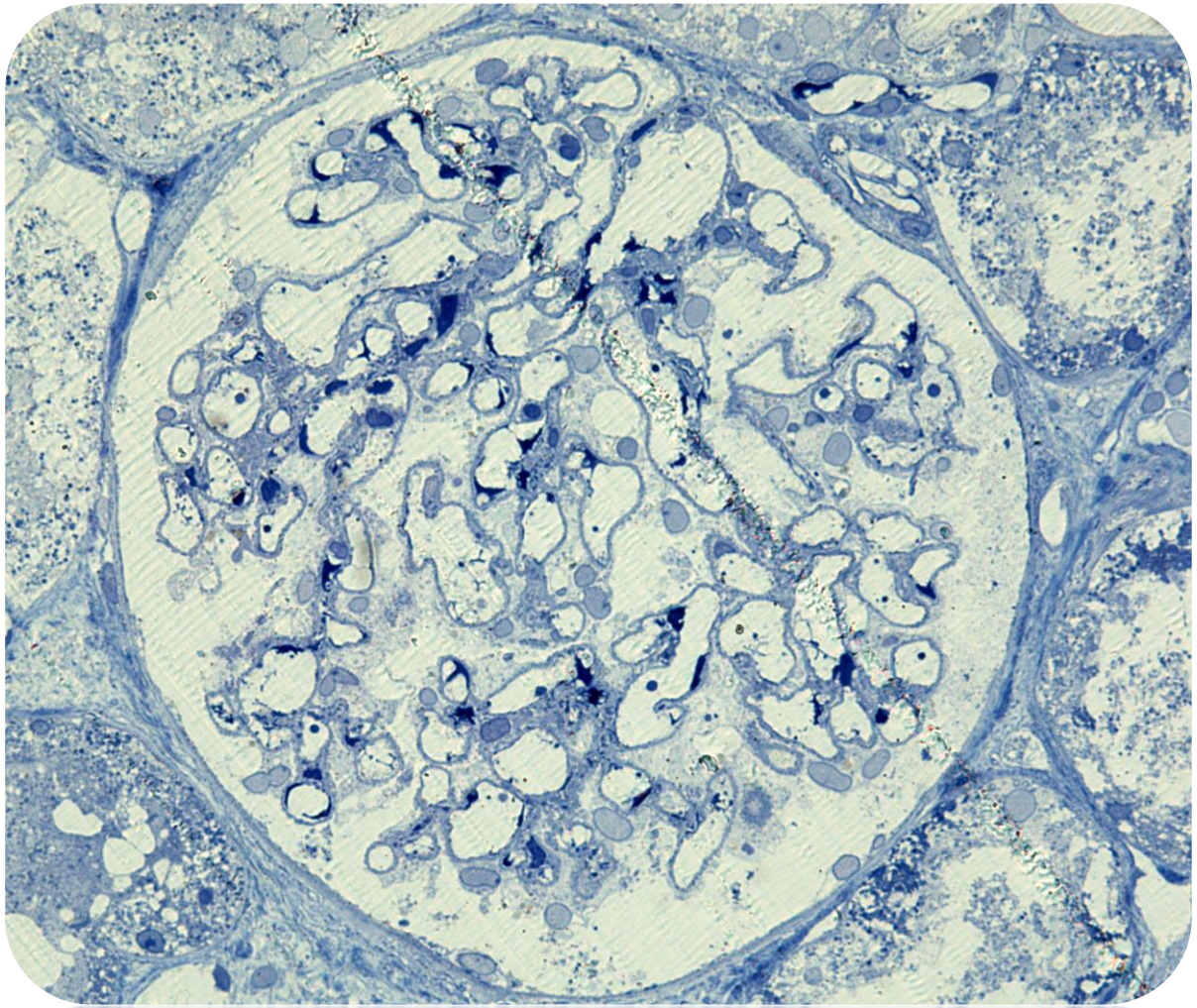
Where have some of the mesangial cells gone? RBC's are also looking different?

Michel's transport medium is excellent for immunofluorescence but in my experience– it's not good for standard EM!!

Is there a different method we could use?

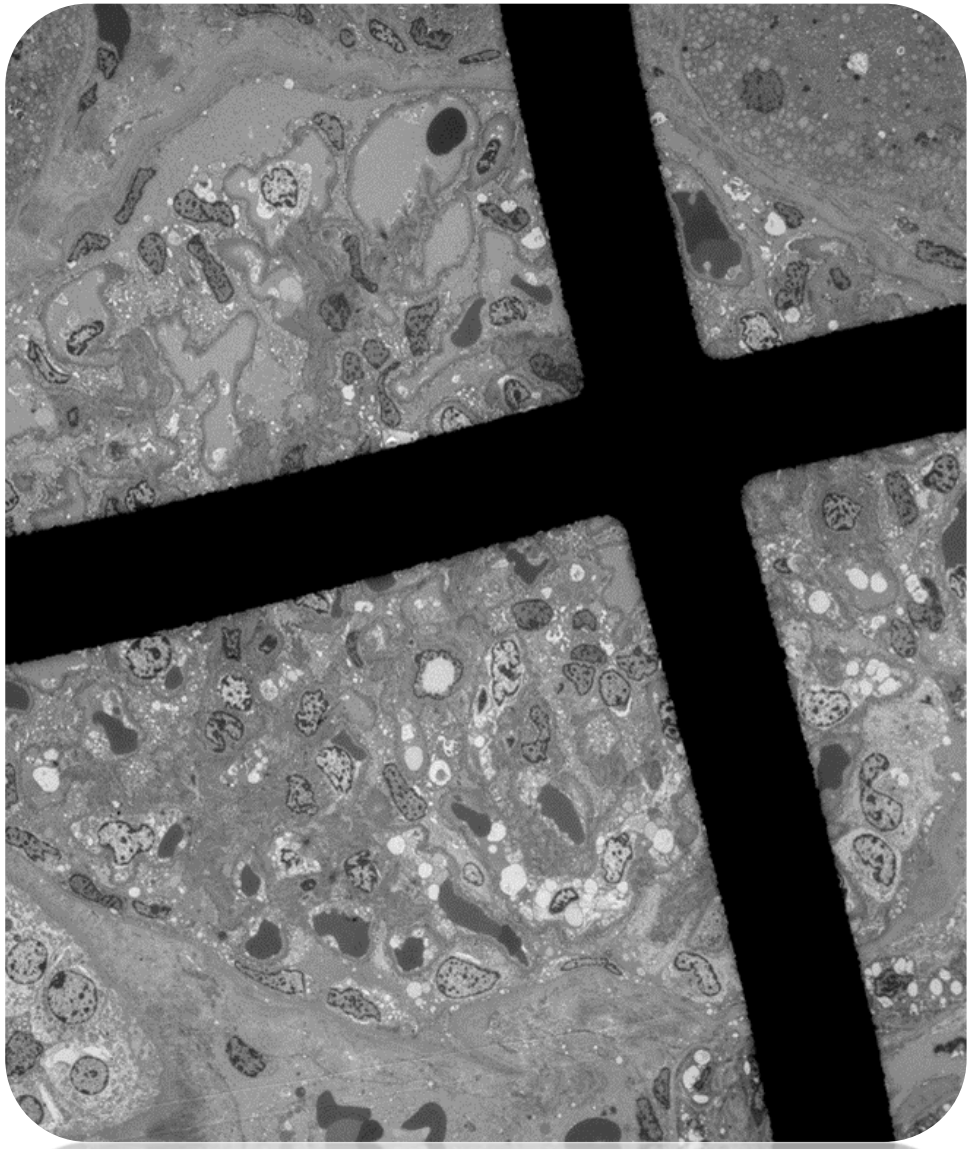


Glutaraldehyde at 5°C

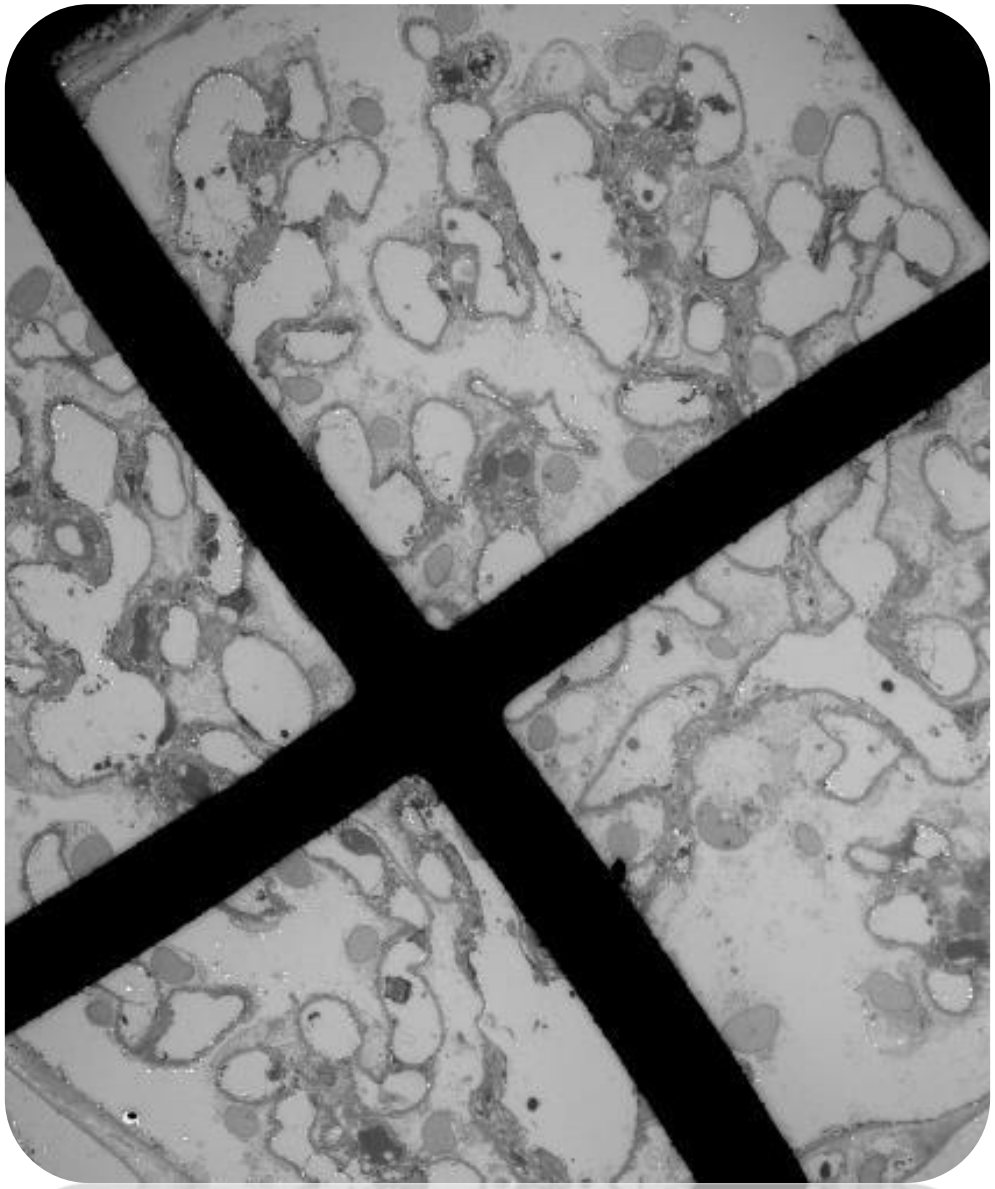


Water

Even worse!

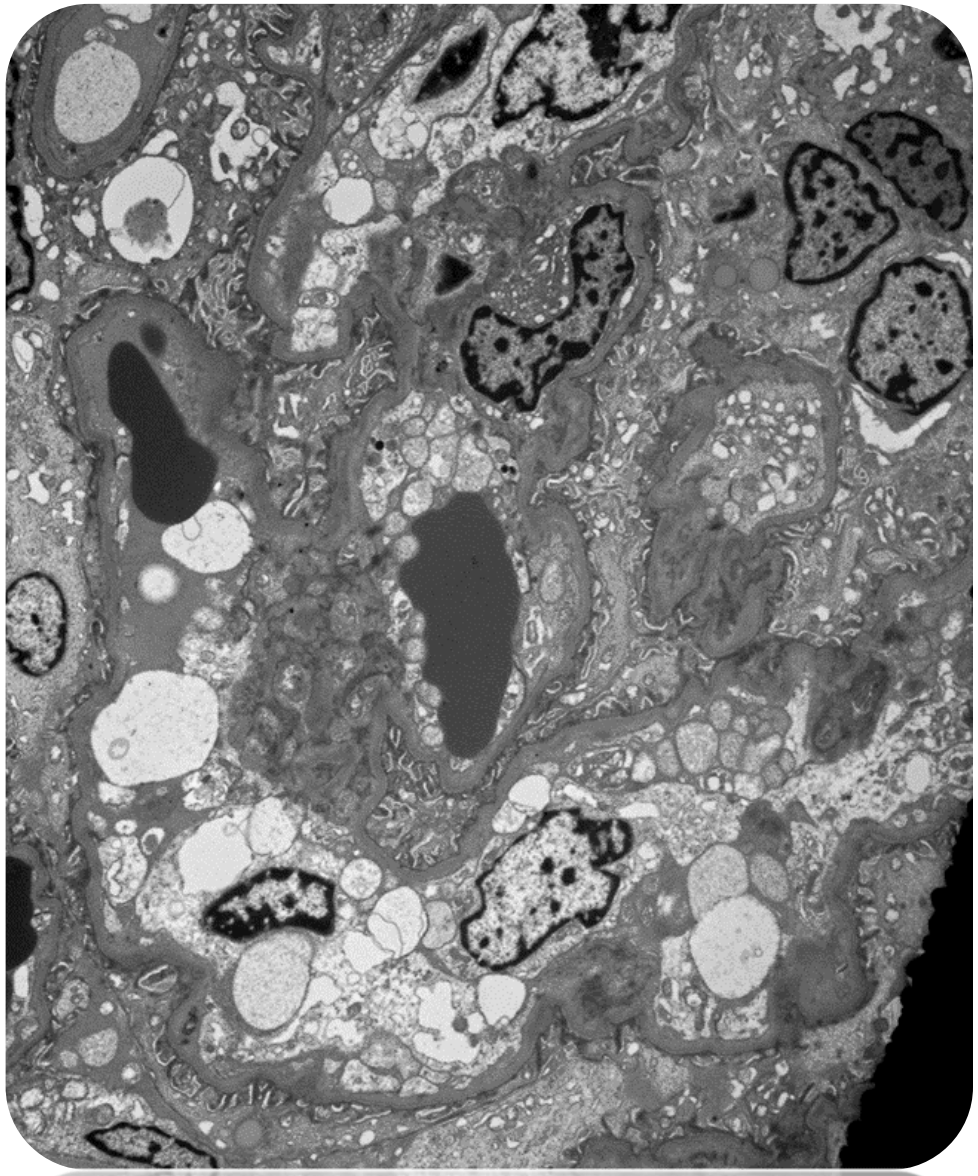


Glutaraldehyde at 5°C

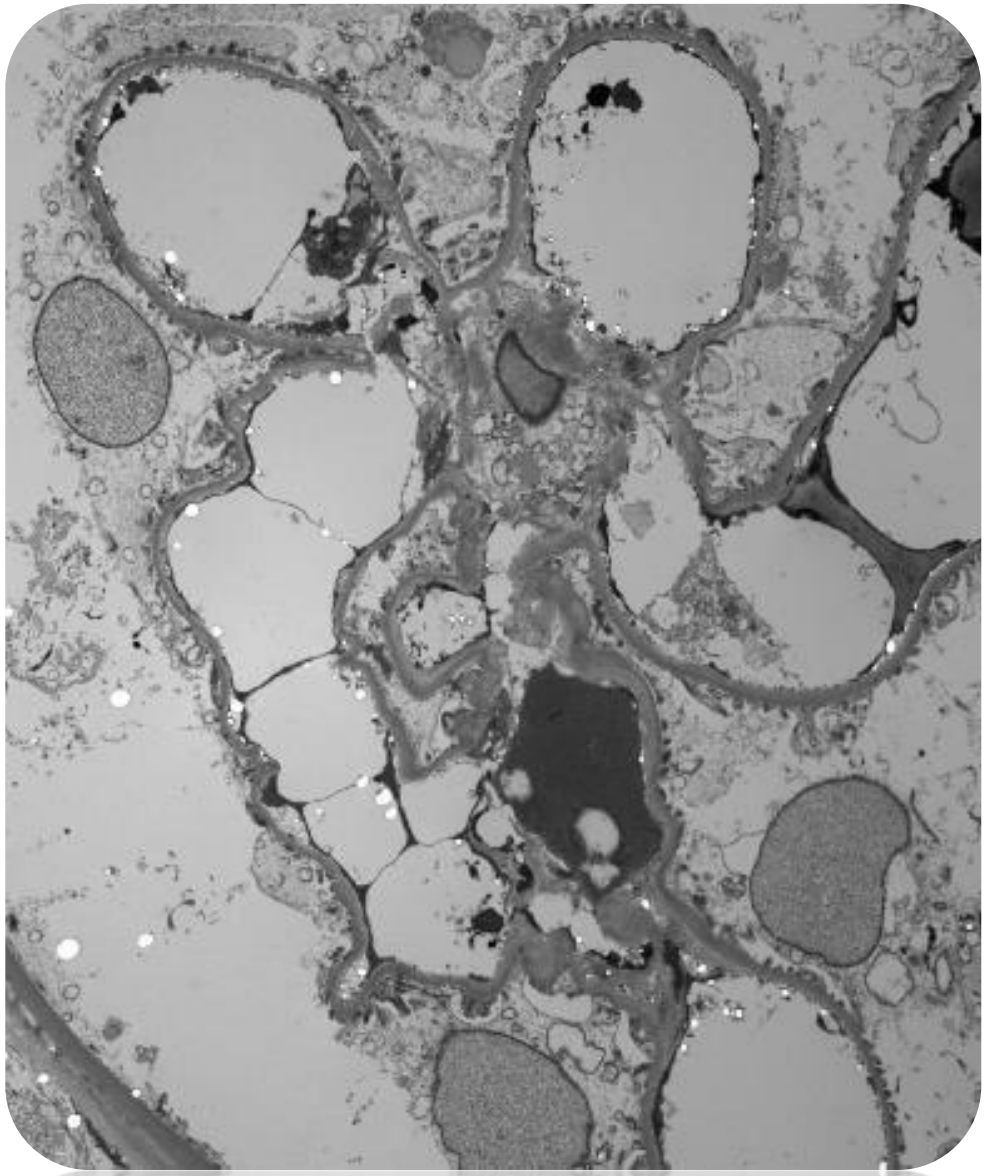


Water

Where have the endothelial cells gone???

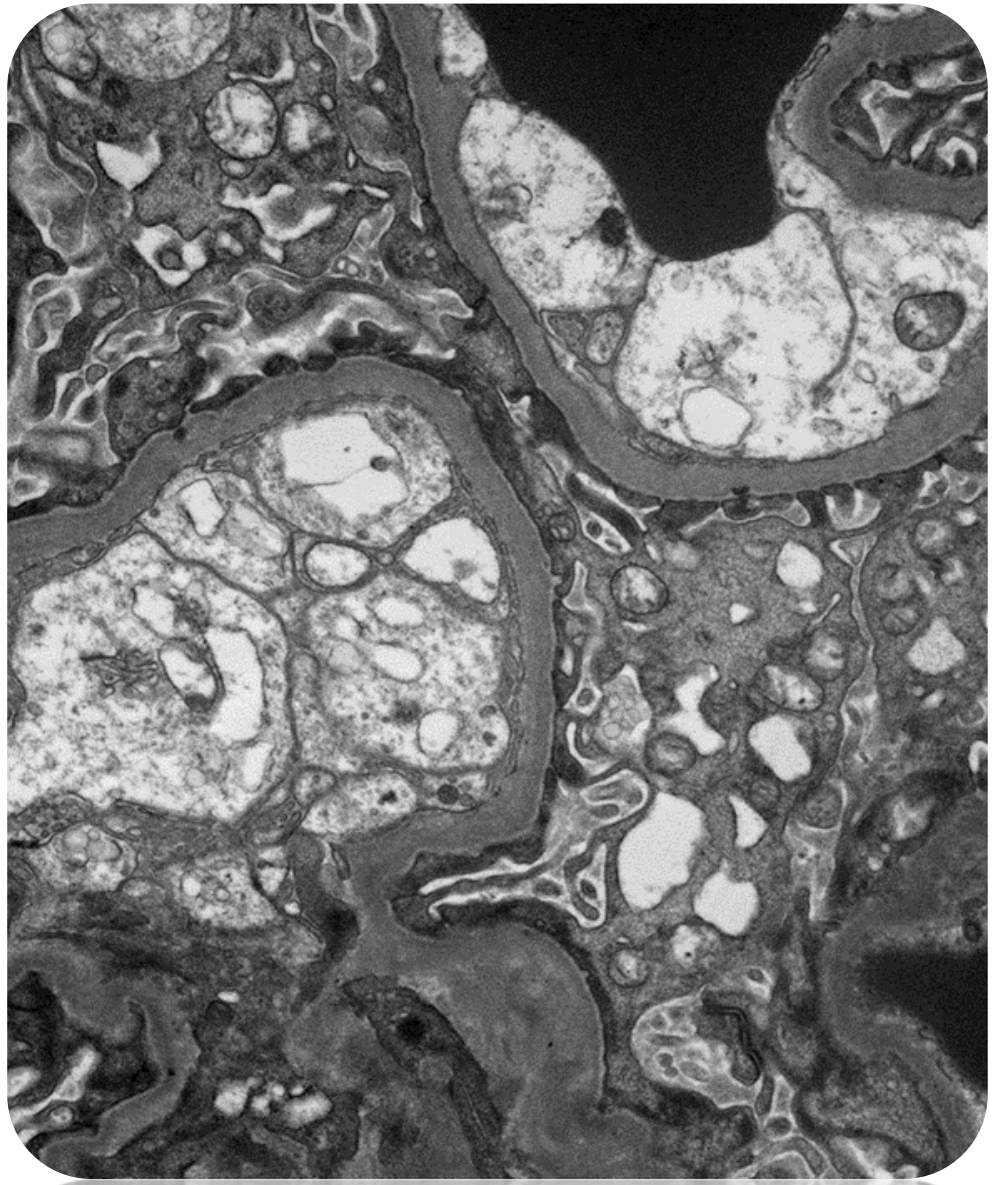


Glutaraldehyde at 5°C

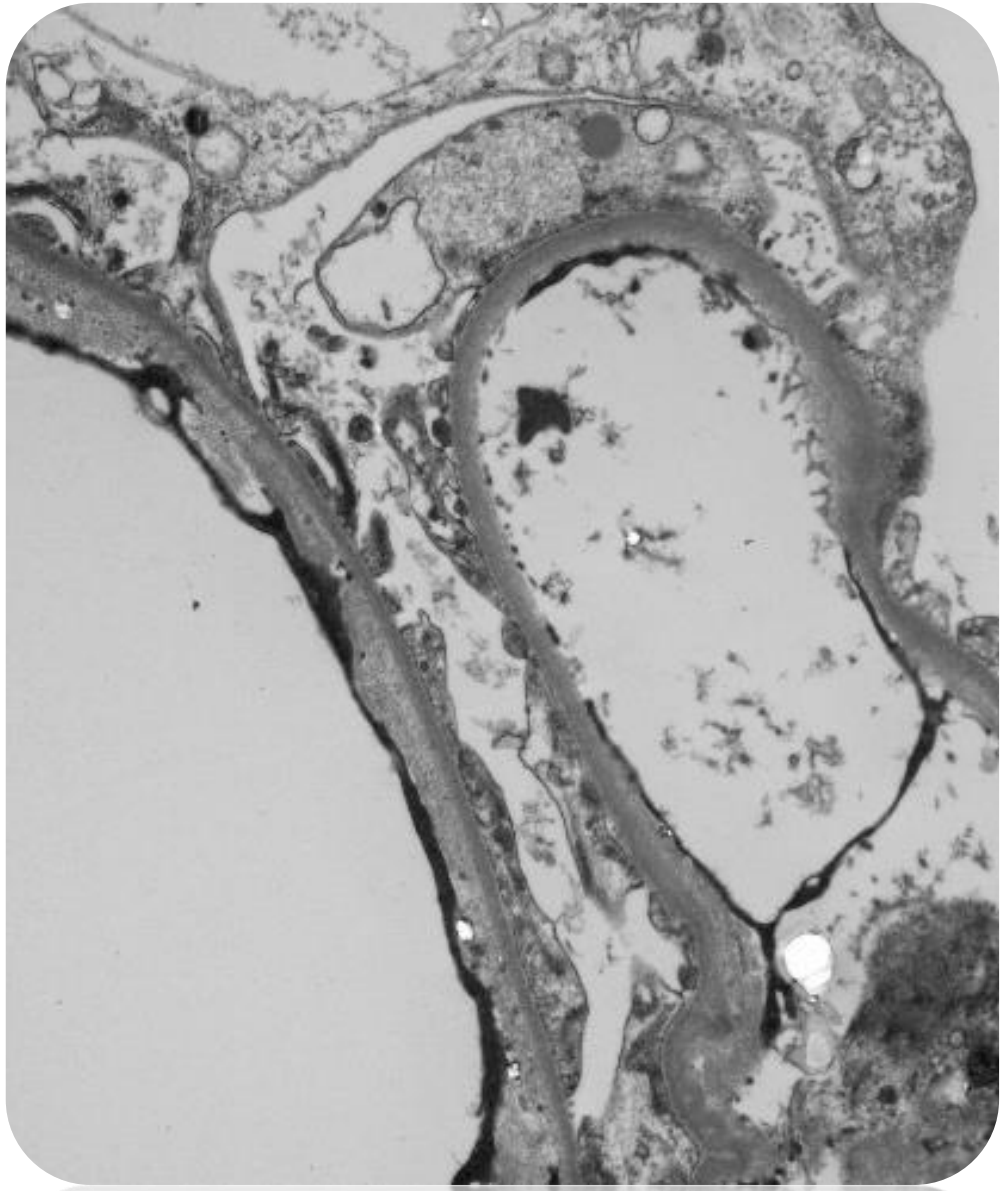


Water

Very poor preservation – extreme loss of soluble proteins and a lot of holes



Glutaraldehyde at 5^{o}C



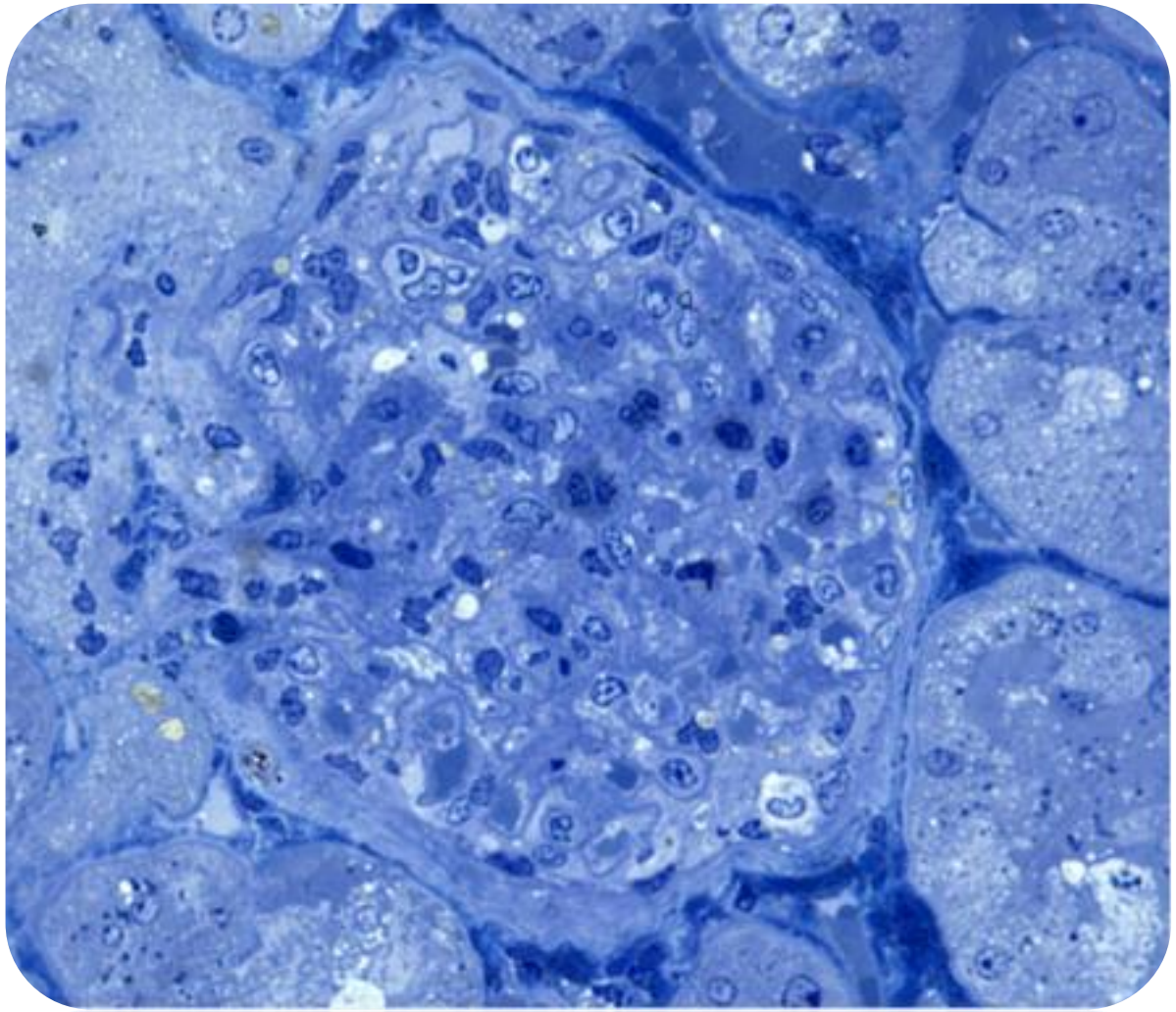
Water

Some of the major problems with this sample include holes in the resin, where processing reagents have not penetrated the tissue sufficiently after fixation. There could be many reasons, but one would be inadequate fixation. Tissue was removed from the patient, sat still in water for 3 hours, then sat still in Glutaraldehyde for 2 hours. Maybe agitation would help?

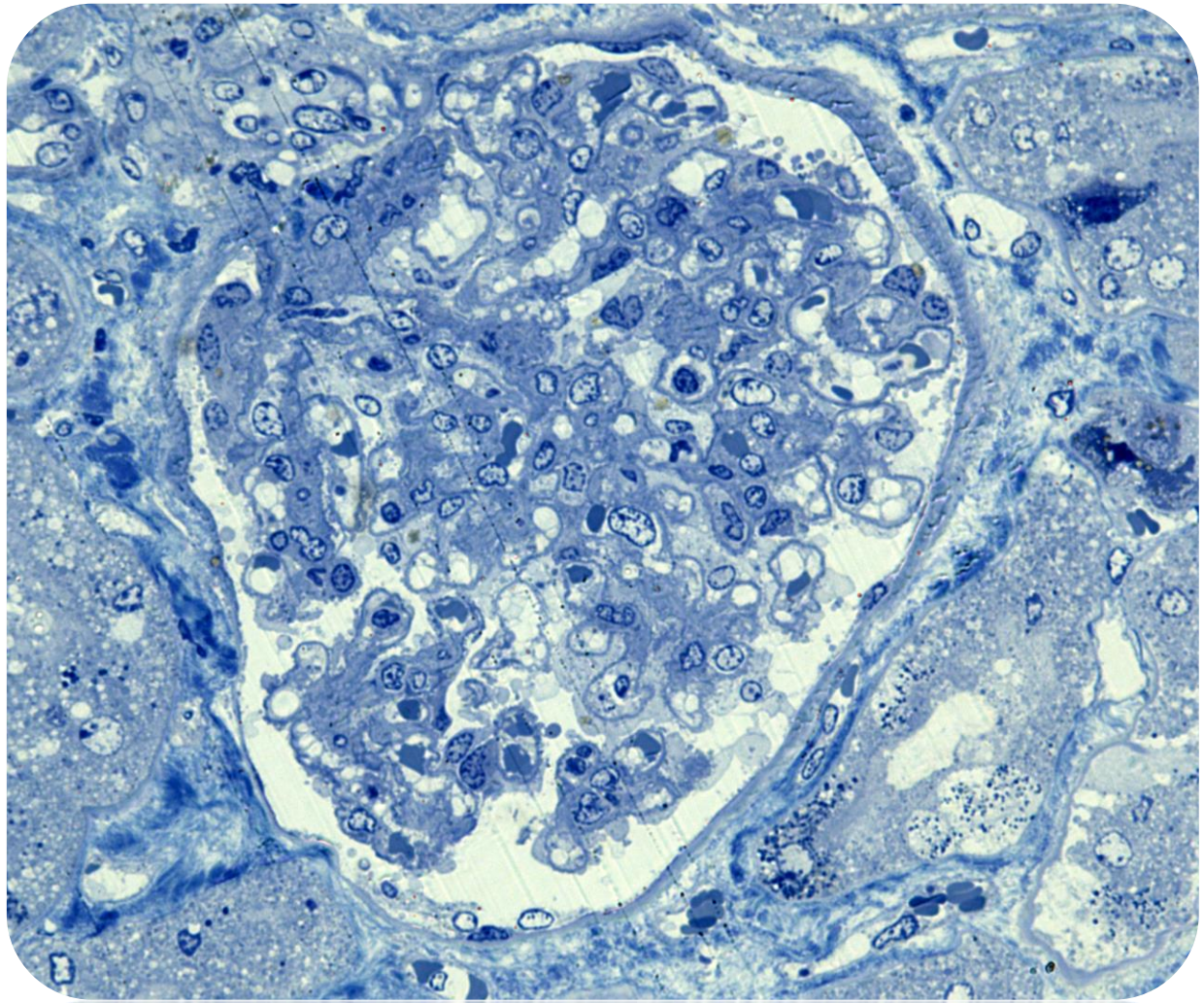
There are no endothelial cells left, and few Red Blood Cells. Are we to assume they have washed away, undergone cytolysis due to the osmosis from the 'un-buffered' water?
We still have centres sending us biopsies in gauze dampened with sterile water instead of saline, which is a marginal improvement at best.

The fenestrae here are continuous and very dark / difficult to visualise

Fuzzy and flat membranes as seen with most of the samples which have suffered delayed fixation of some sort.

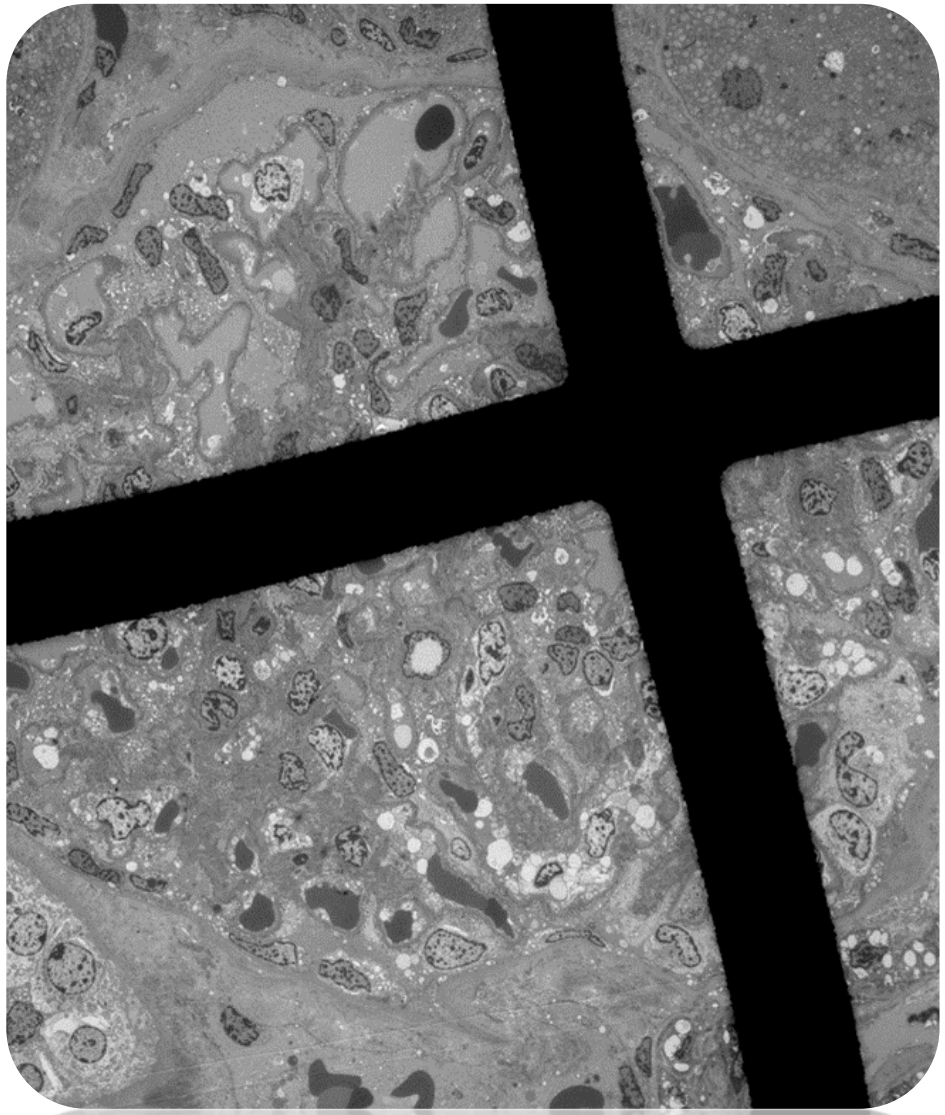


Glutaraldehyde at 5°C

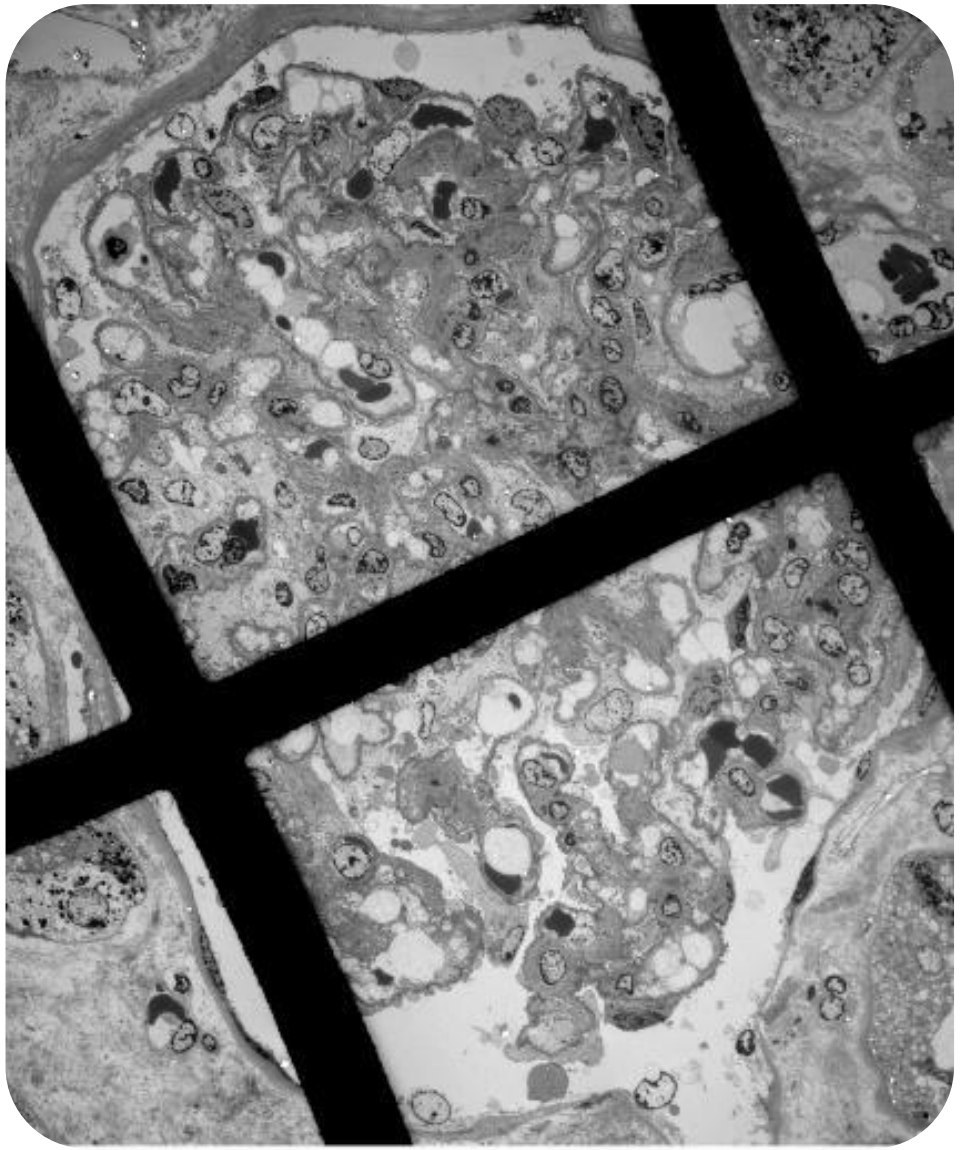


Phosphate Buffered Saline

Quite a gap between glom and capsule again…

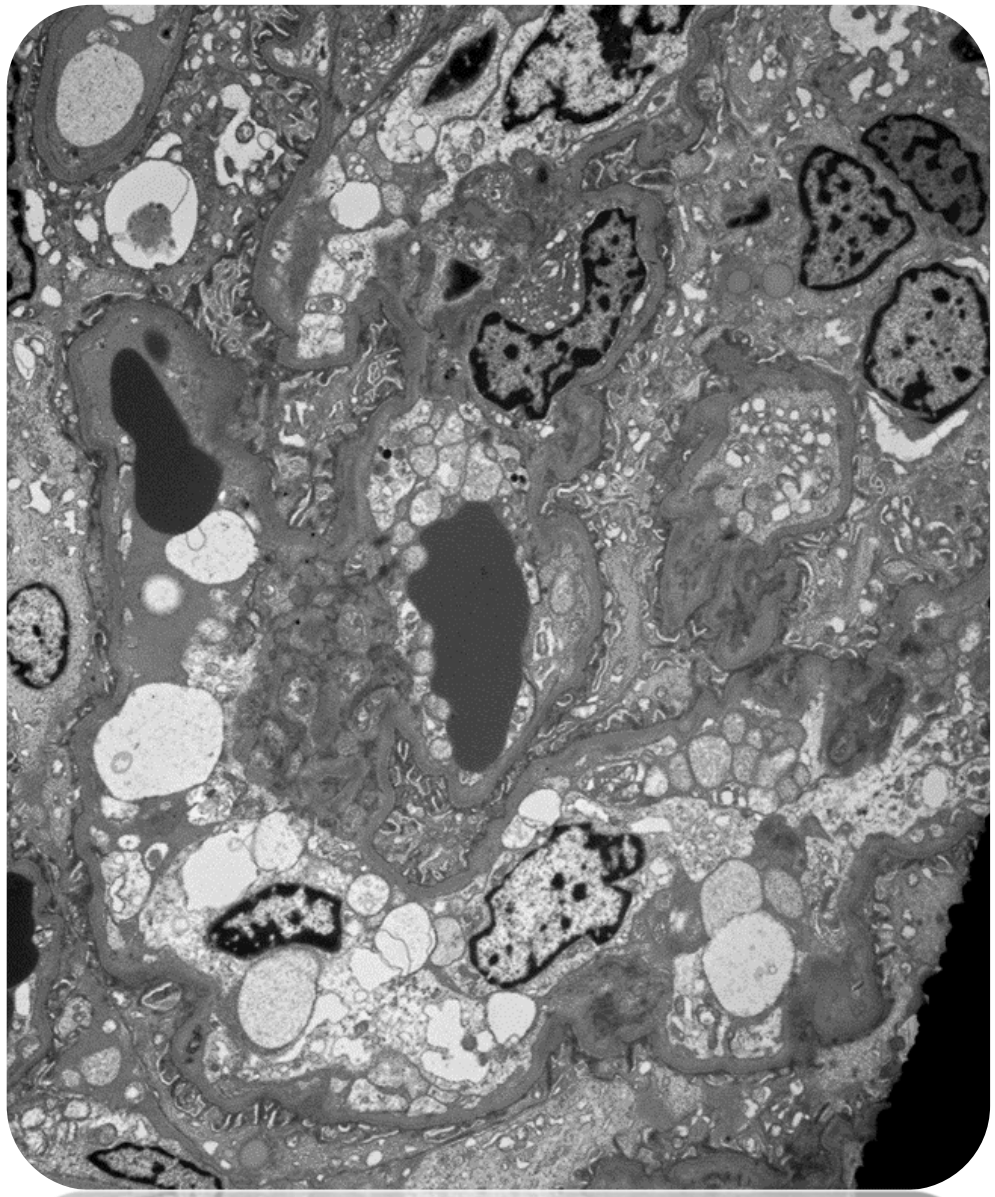


Glutaraldehyde at 5°C

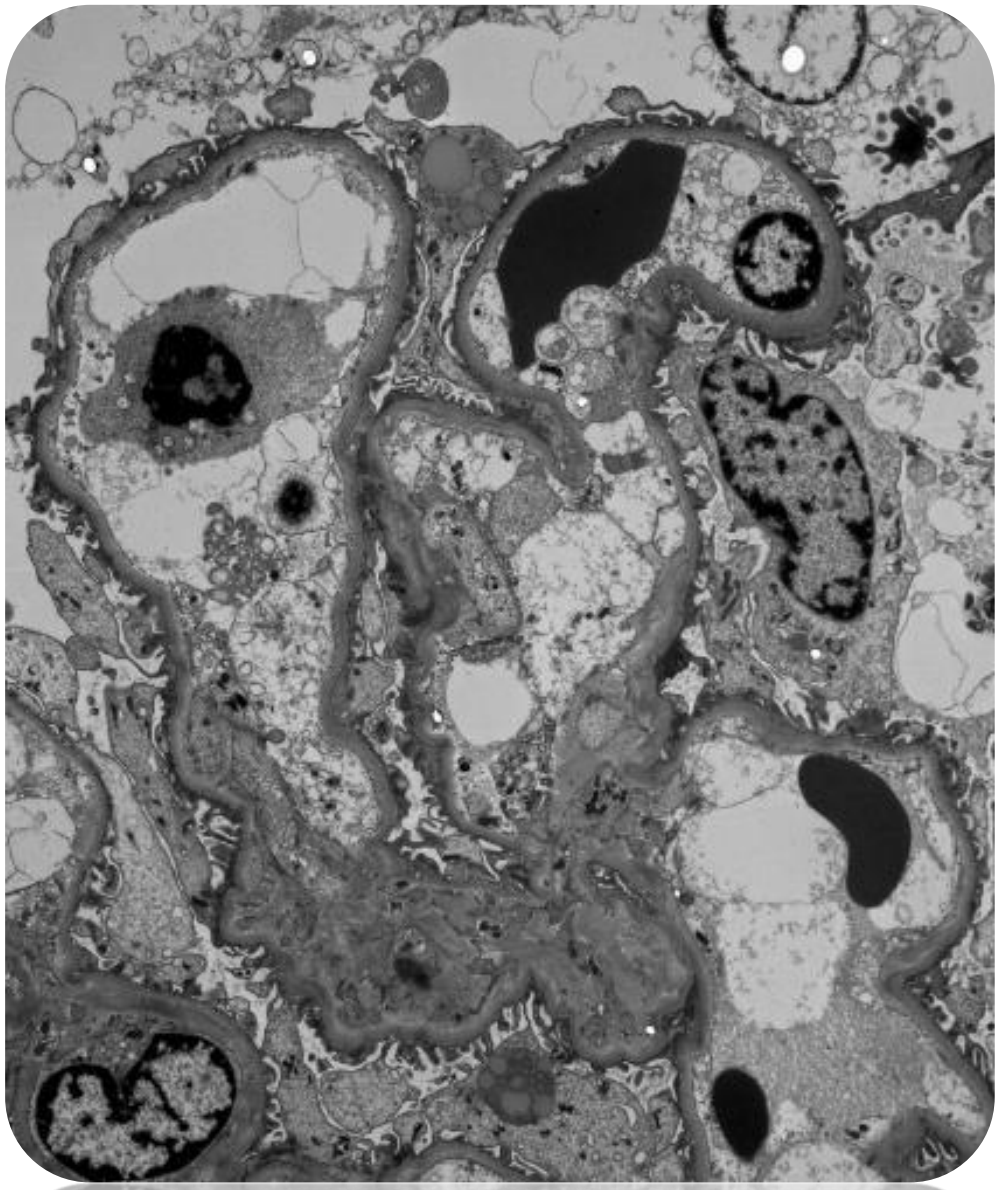


Phosphate Buffered Saline

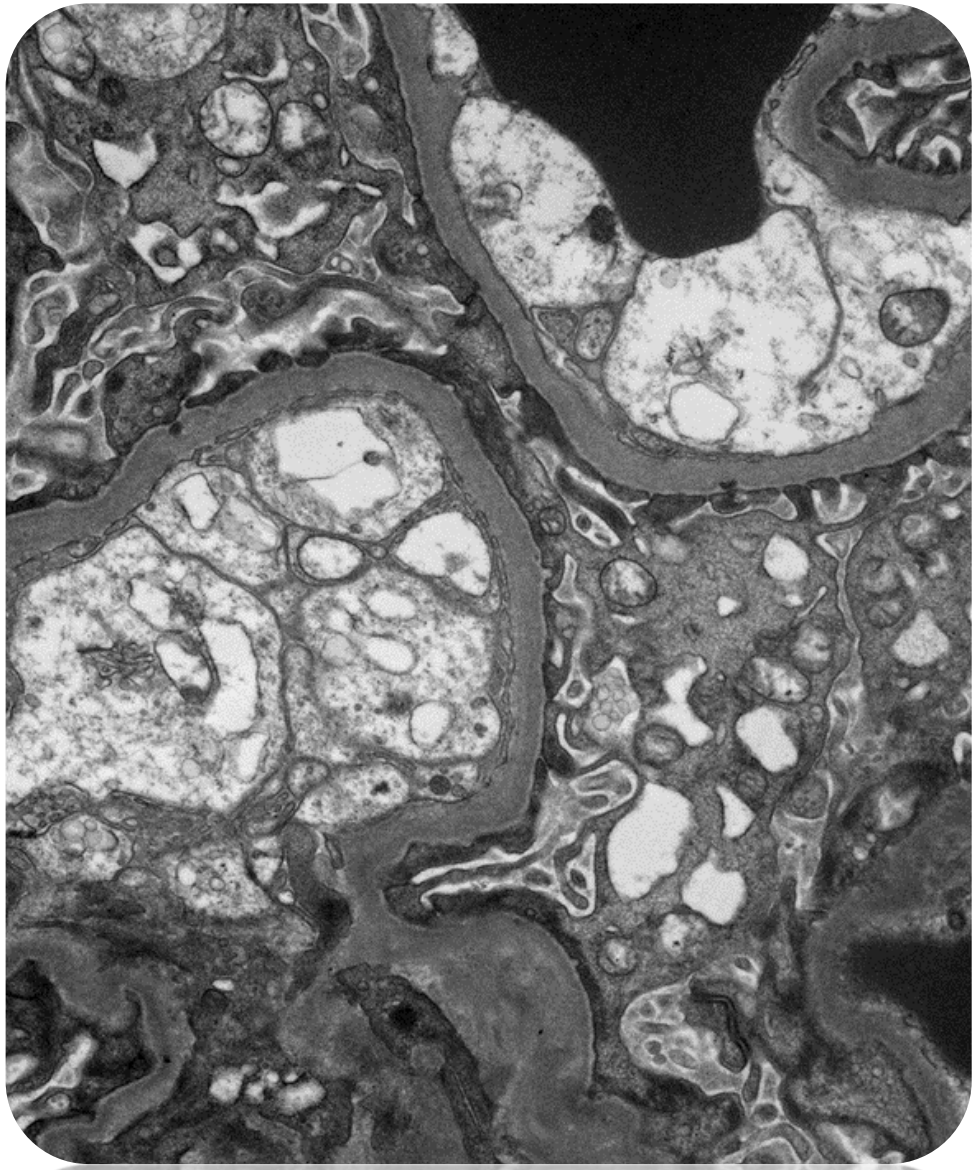
Small holes in the resin – more issues with processing from questionably fixed samples?



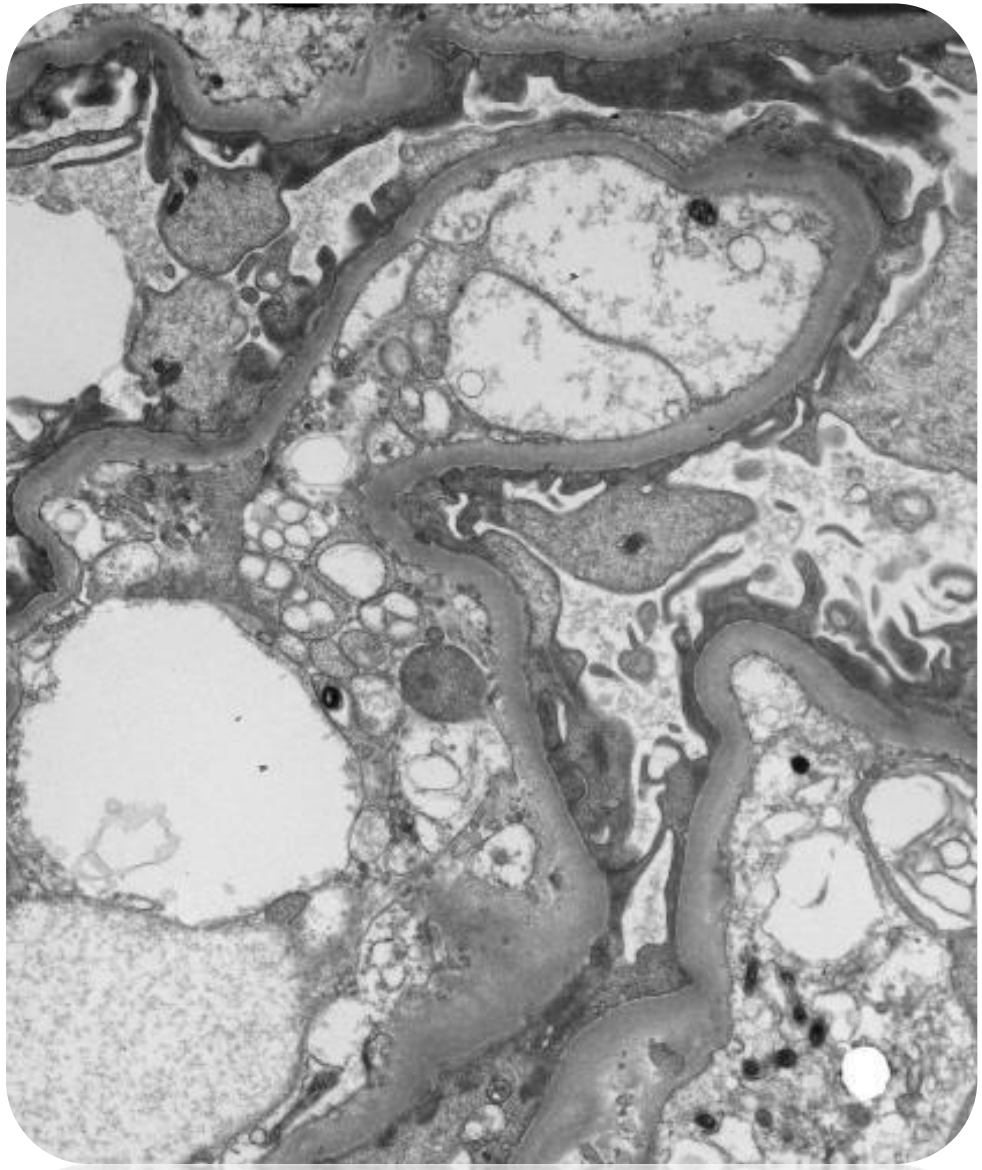
Glutaraldehyde at 5°C



Phosphate Buffered Saline



Glutaraldehyde at 5°C



Phosphate Buffered Saline

Some of those soluble proteins have definitely been lost. Our biopsy is pale, but our mitochondria are better preserved

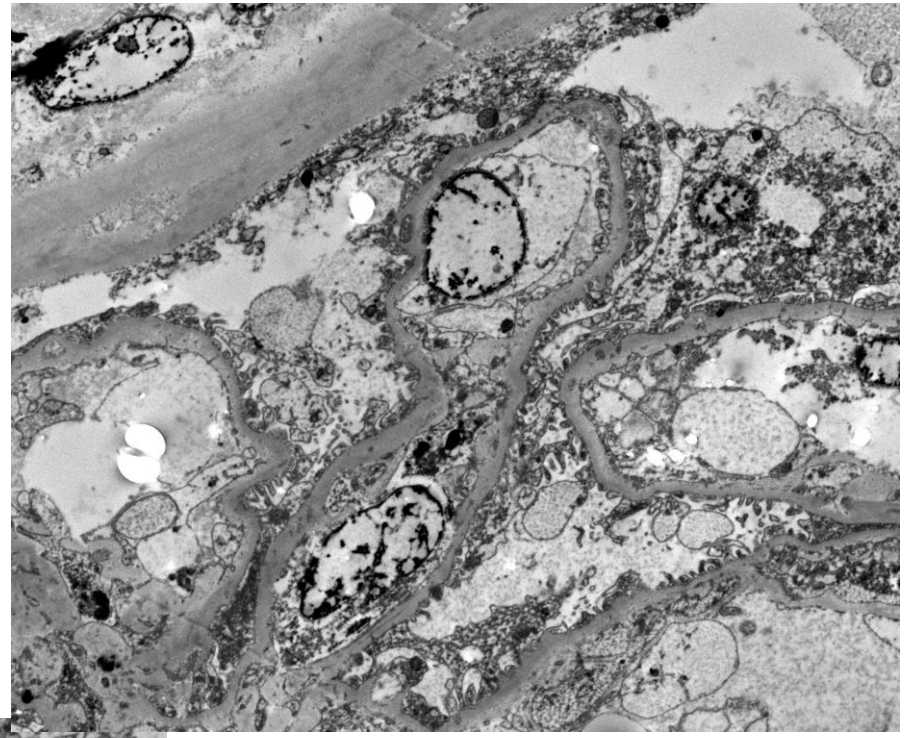
Similar to the water immersed sample, staining is flat and the membranes appear quite fuzzy
There is again a lack of cytoplasmic details and shrinkage of the capillaries away from Bowman's capsule.

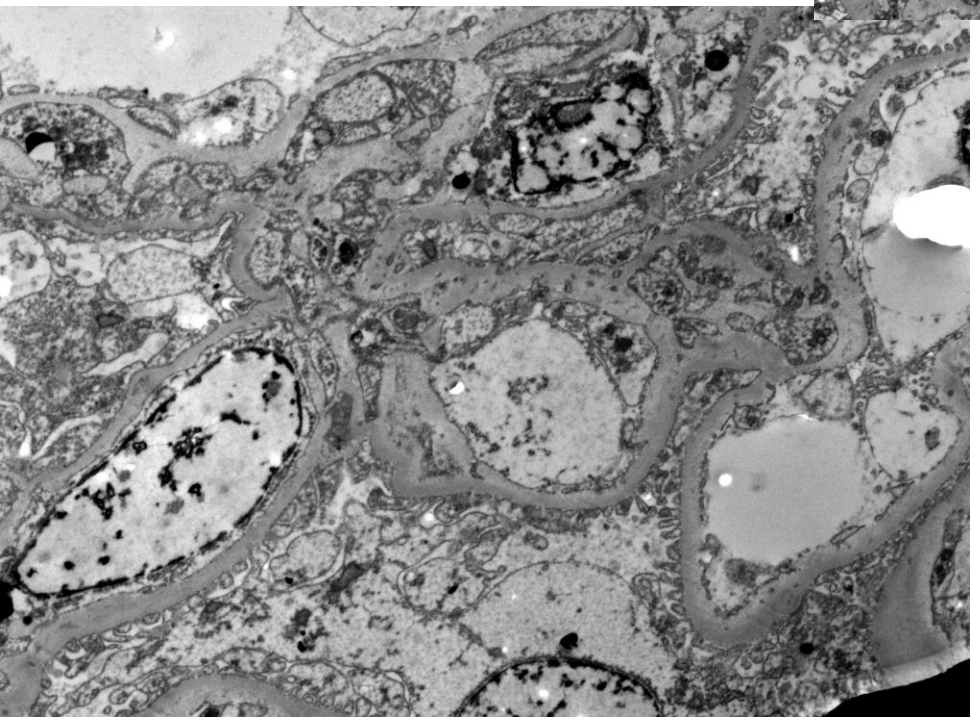
The Podocyte disruption is again quite obvious however, we do have some retention of the podocytes and their foot processes.

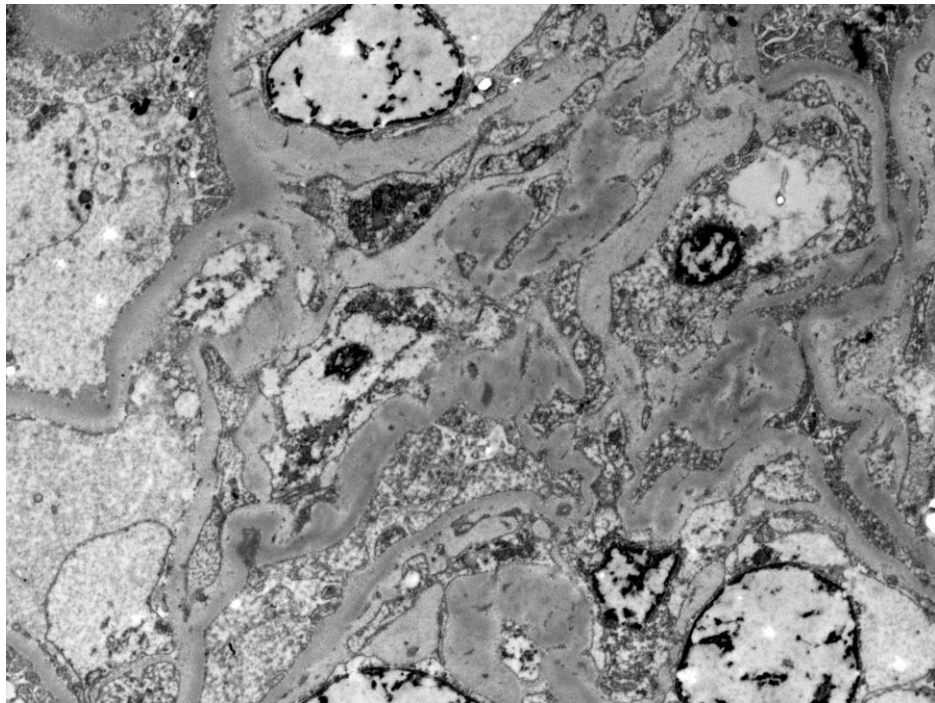
Again, we can see a lack of fenestrae, but there appears to be some areas where they are preserved.

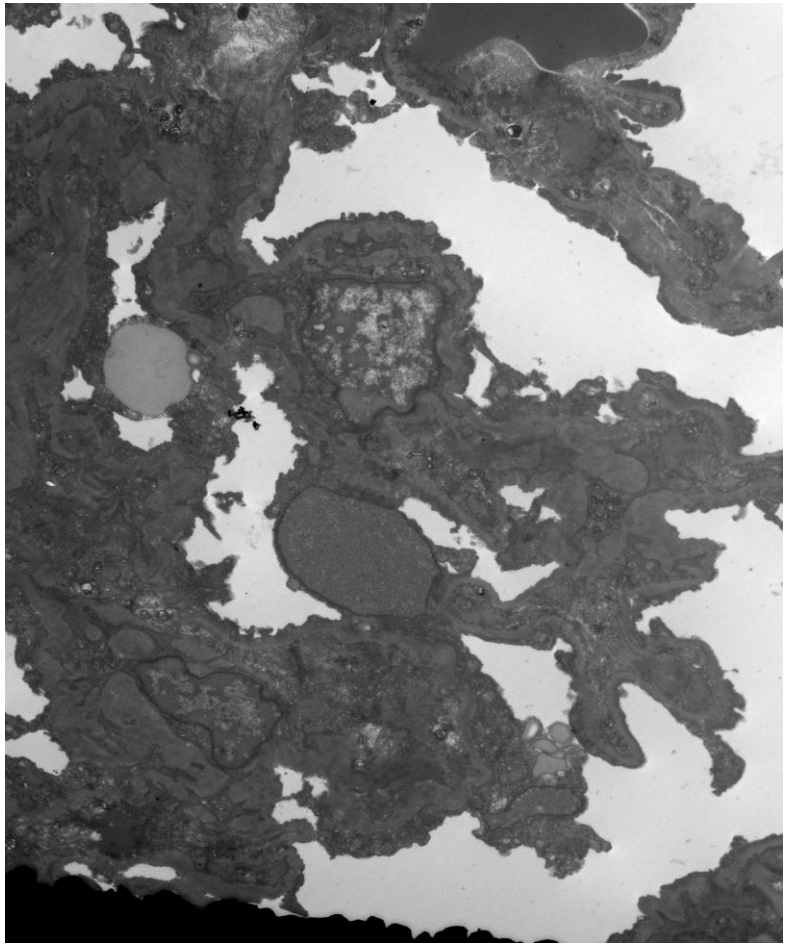
Although we don't have any toluidine blue images, the next few slides contain Electron micrographs of kidney biopsies
Where the PBS has been made up wrong, producing a different pH and concentration of the solution to that usually used.

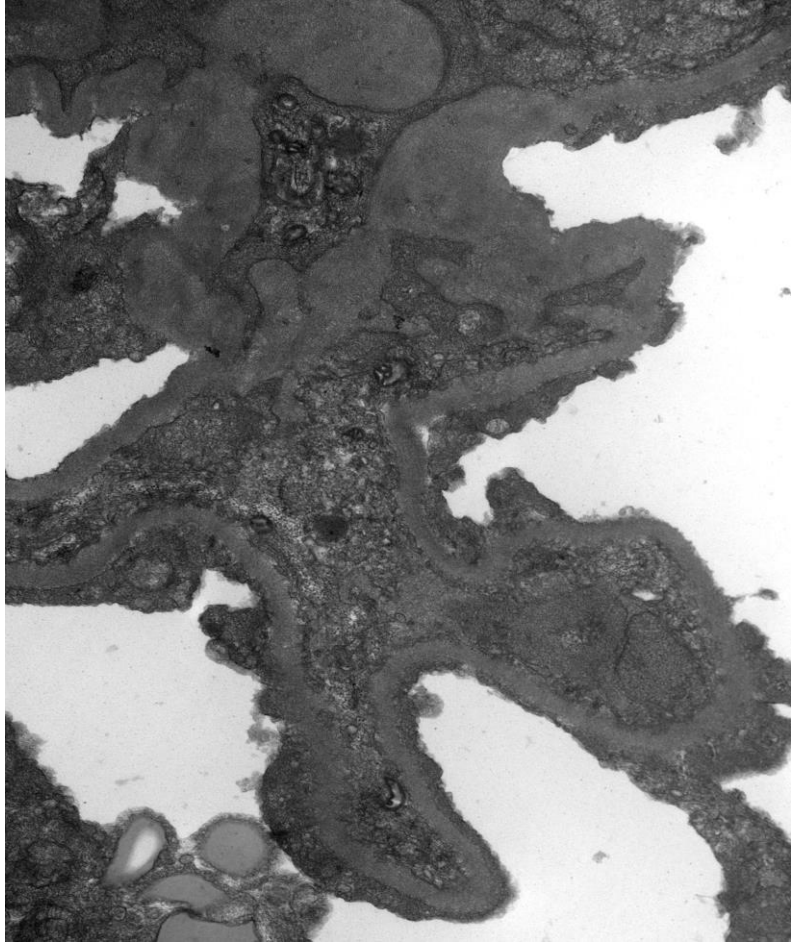
Prepare yourselves !

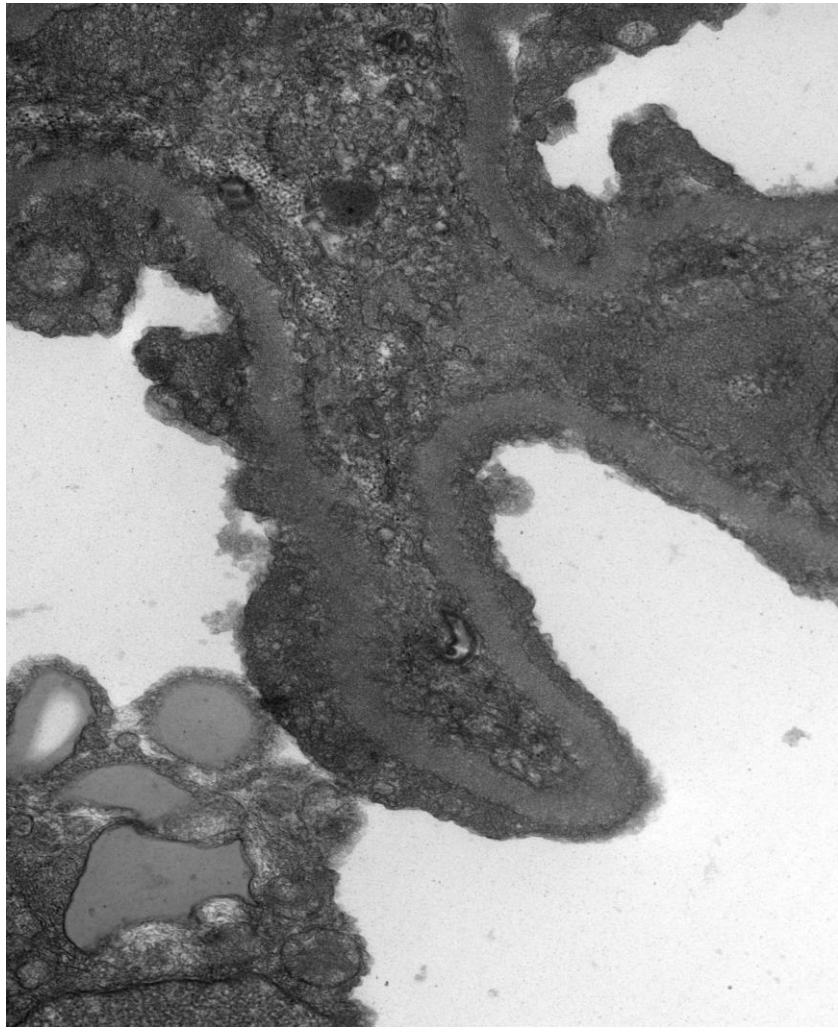








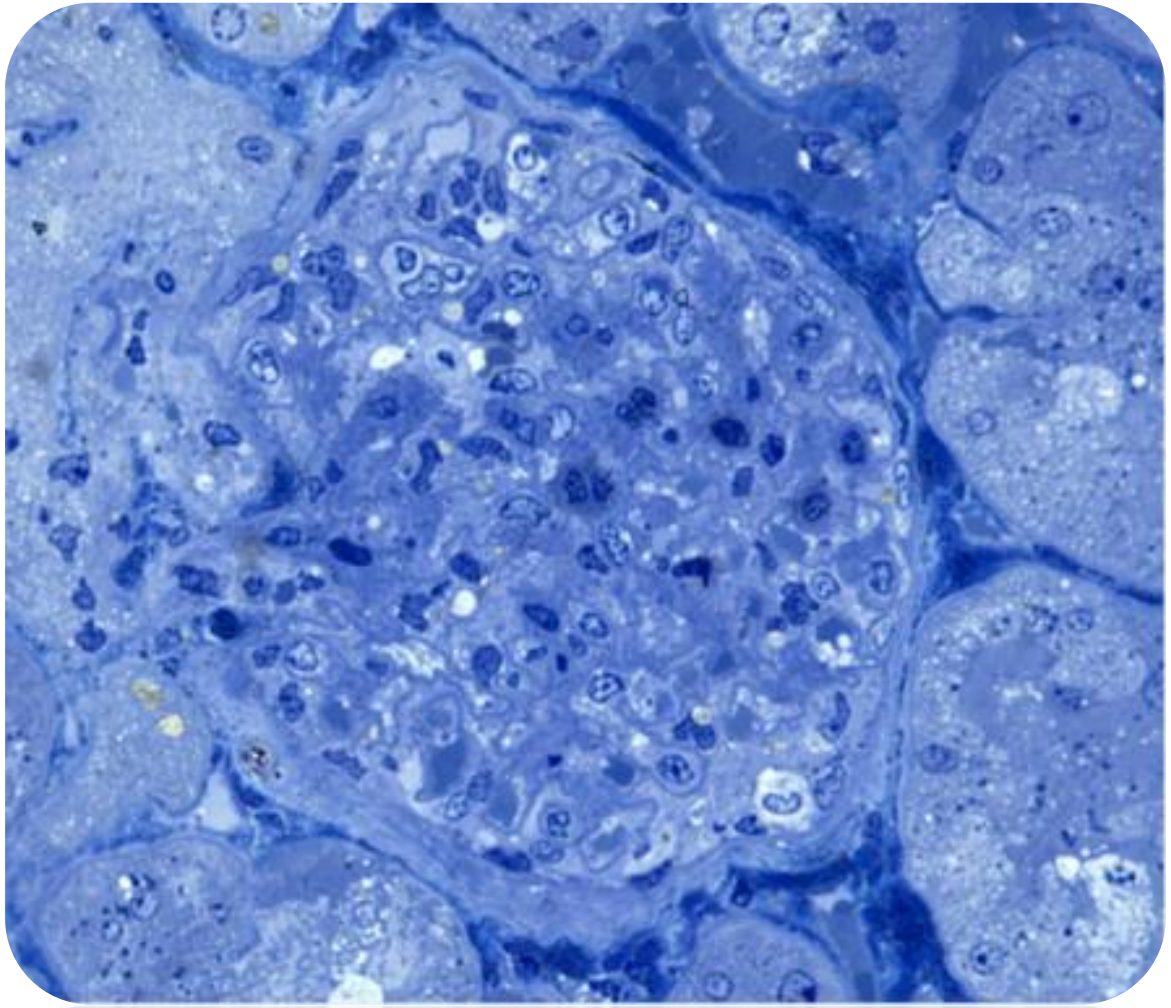




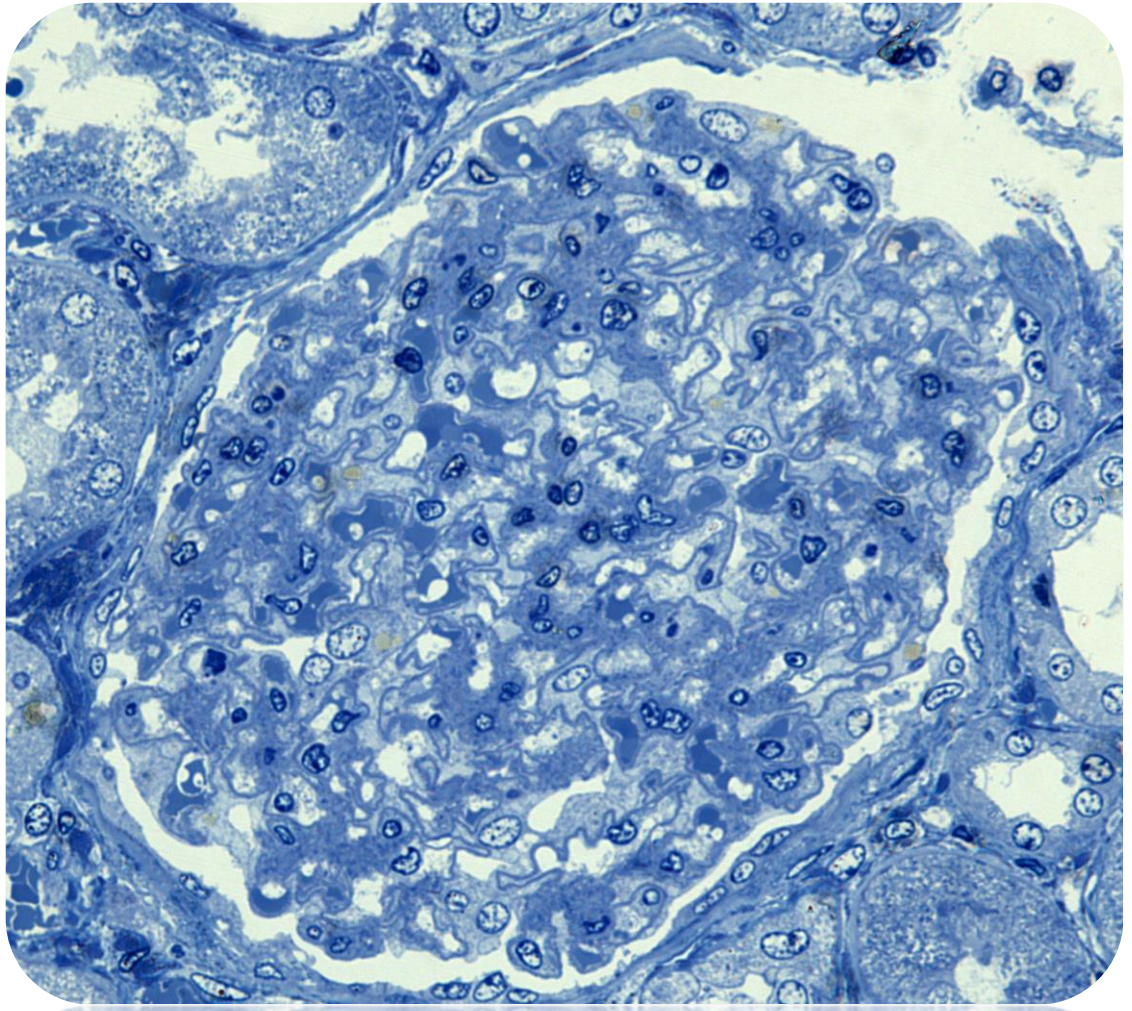
Many thanks to Natalie Wilson and Tracey De Haro for the additional images

I'm certain we can all agree that this has rendered the tissue un-screenable - although Tracey and Natalie's tissue seems to have survived a little better than mine.

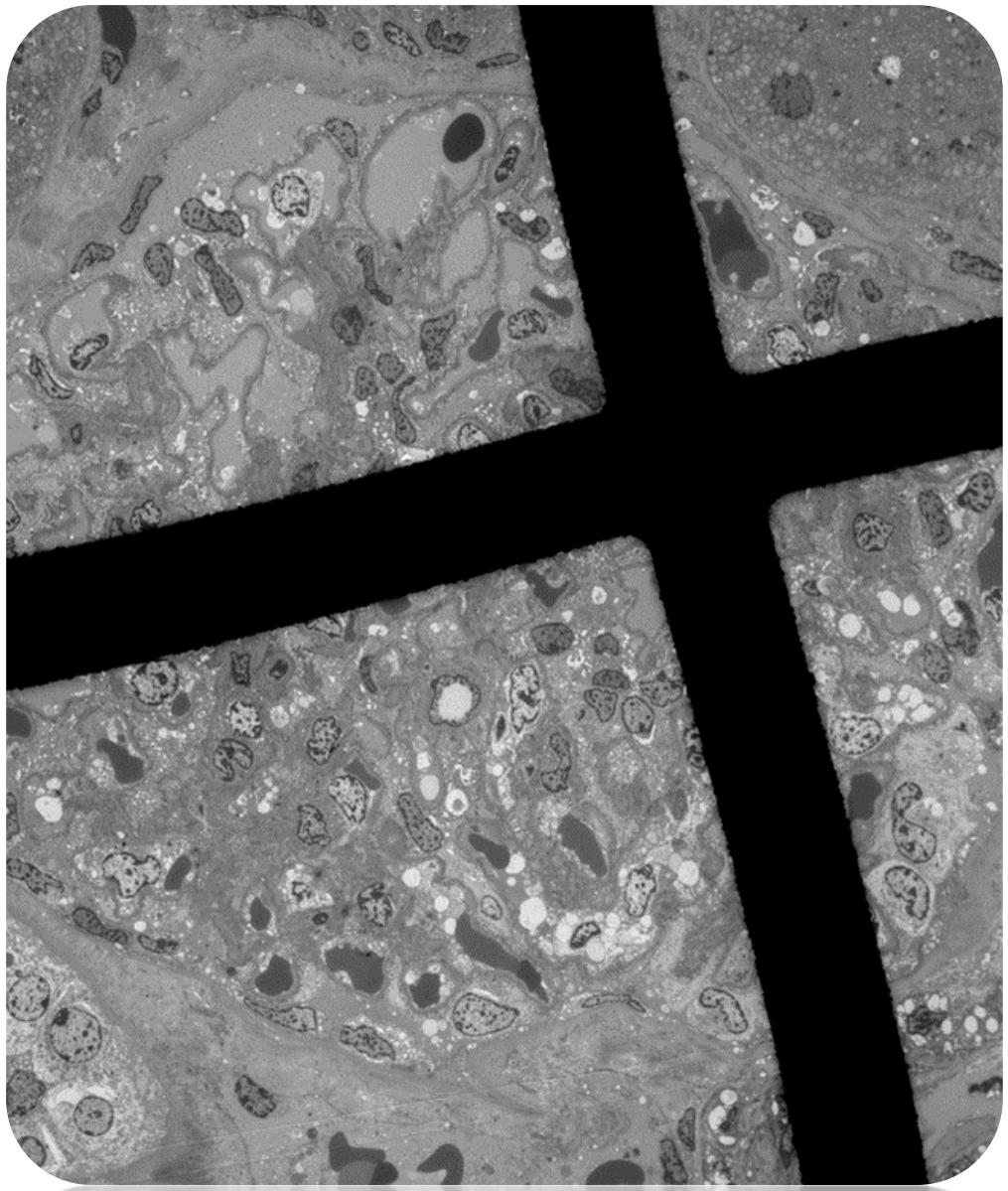
The pH of the solution is very important in maintaining tissue morphology -
Please don’t ask me how...



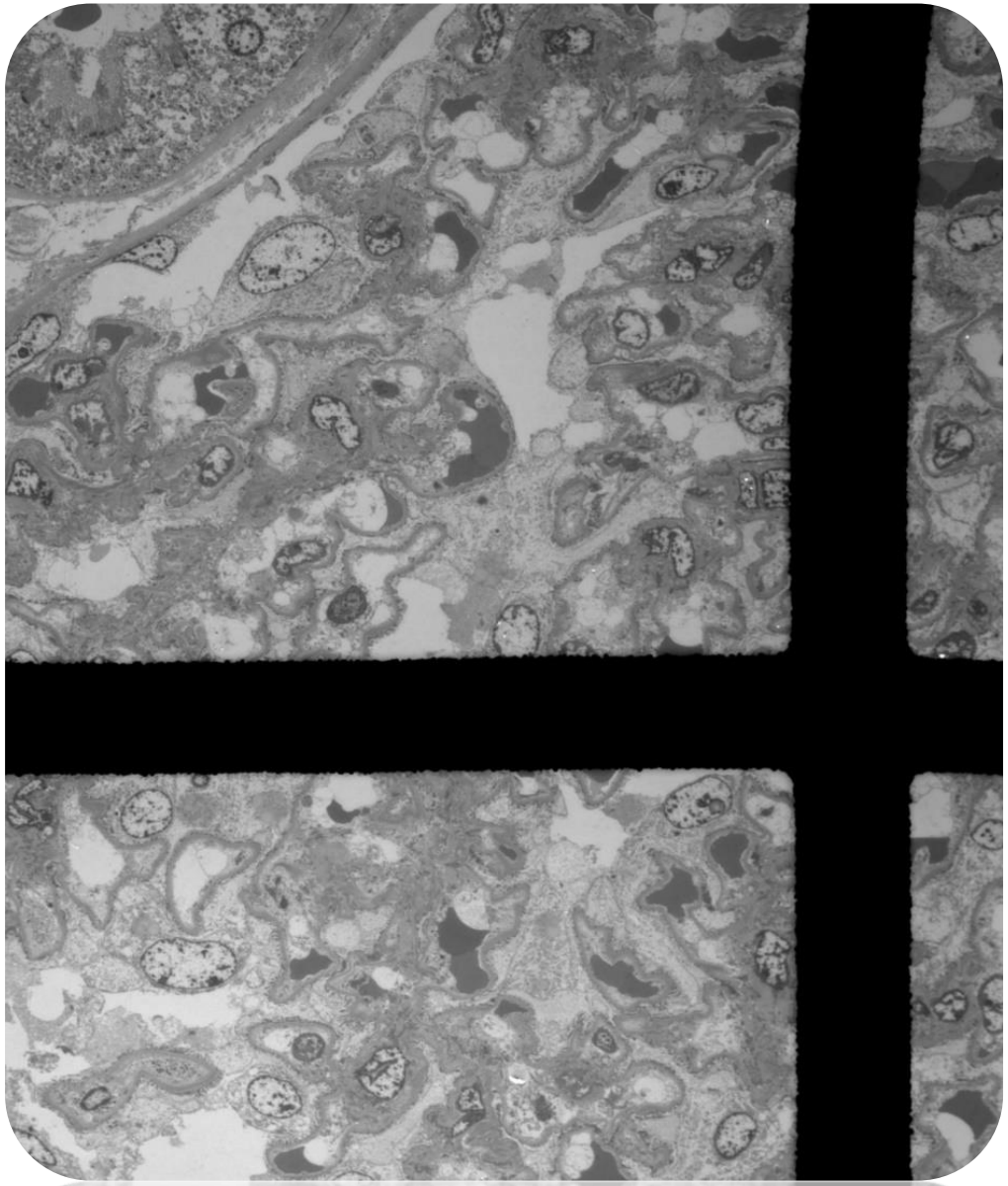
Glutaraldehyde at 5°C



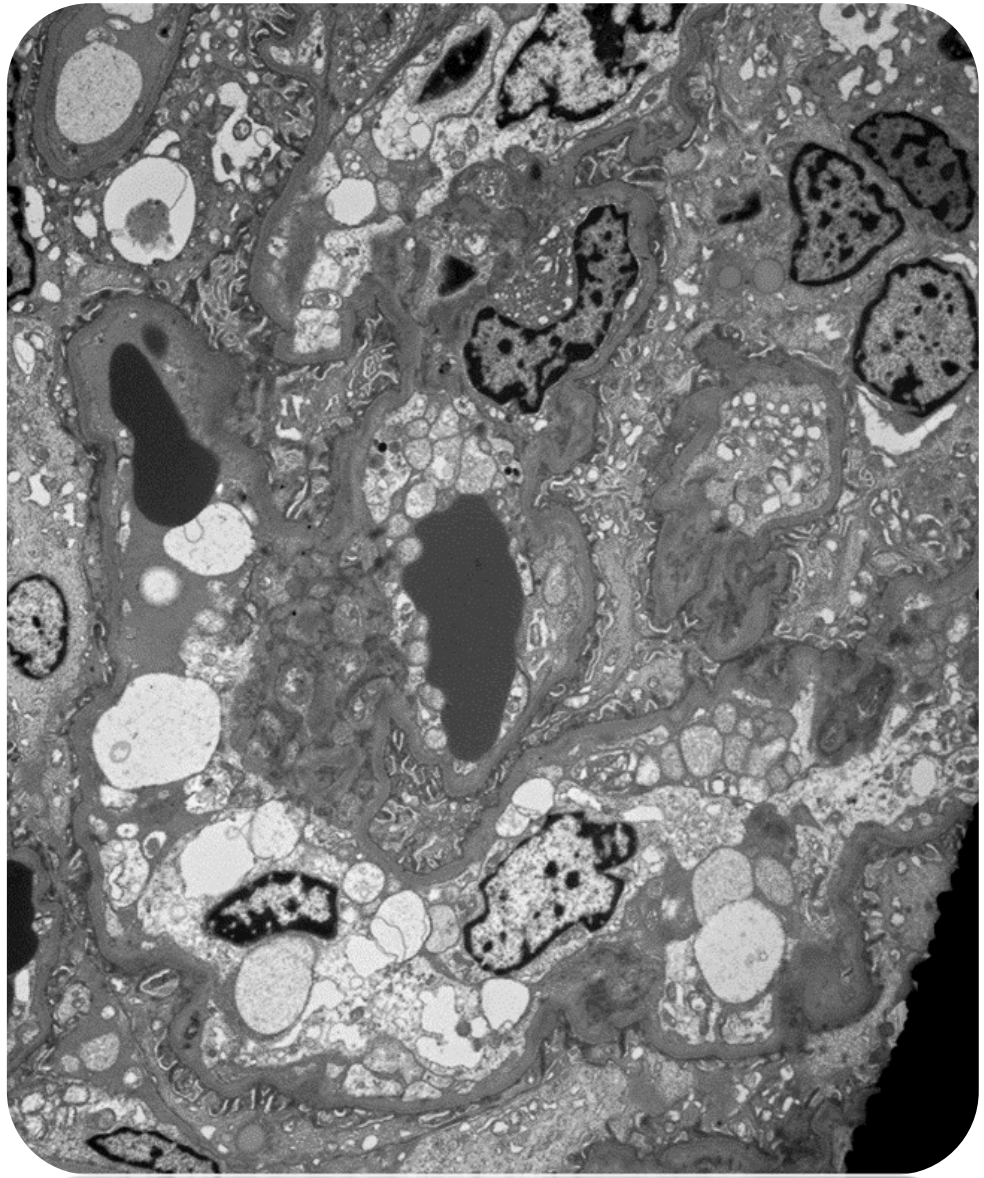
Long term storage



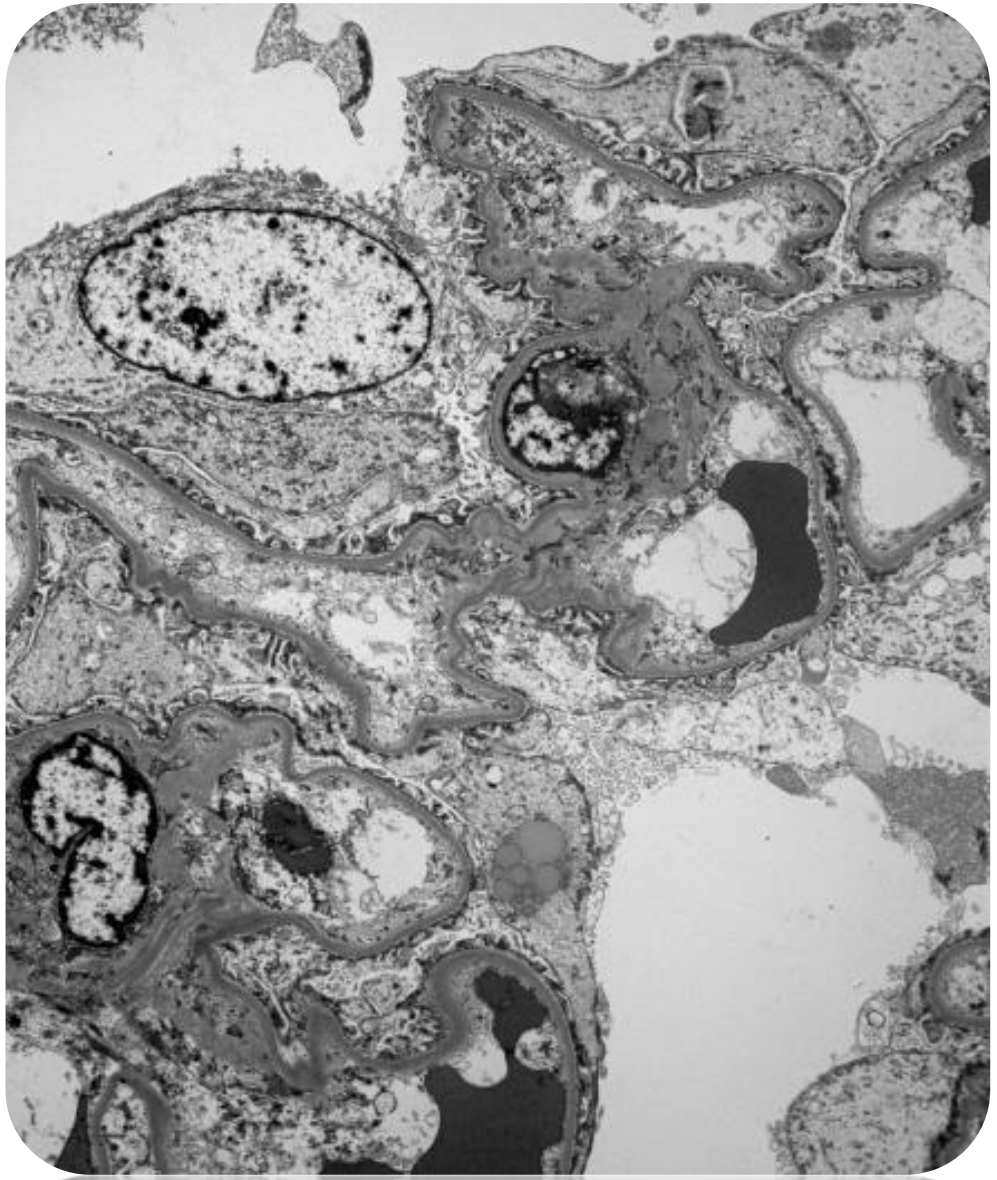
Glutaraldehyde at 5°C



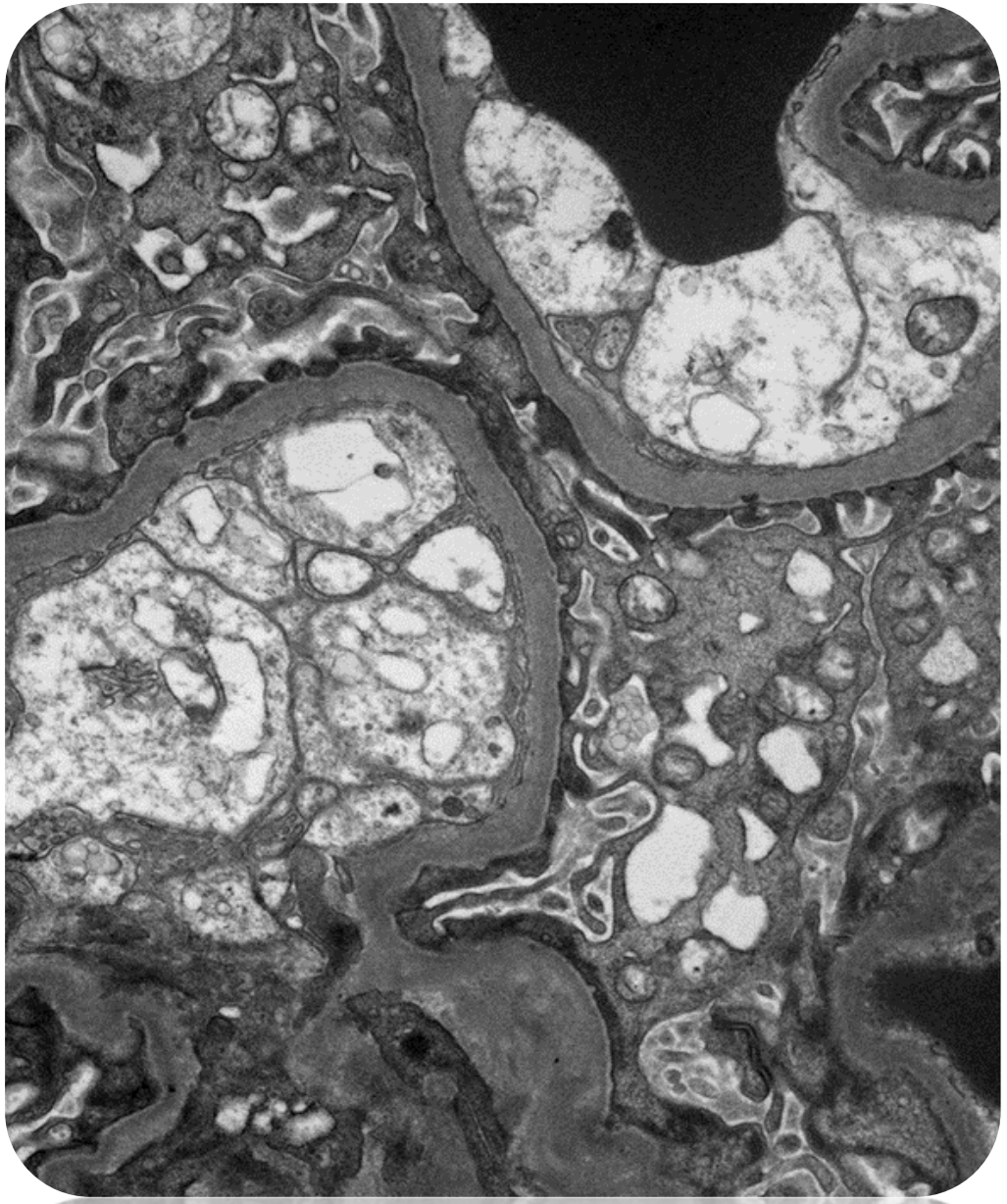
Long term storage



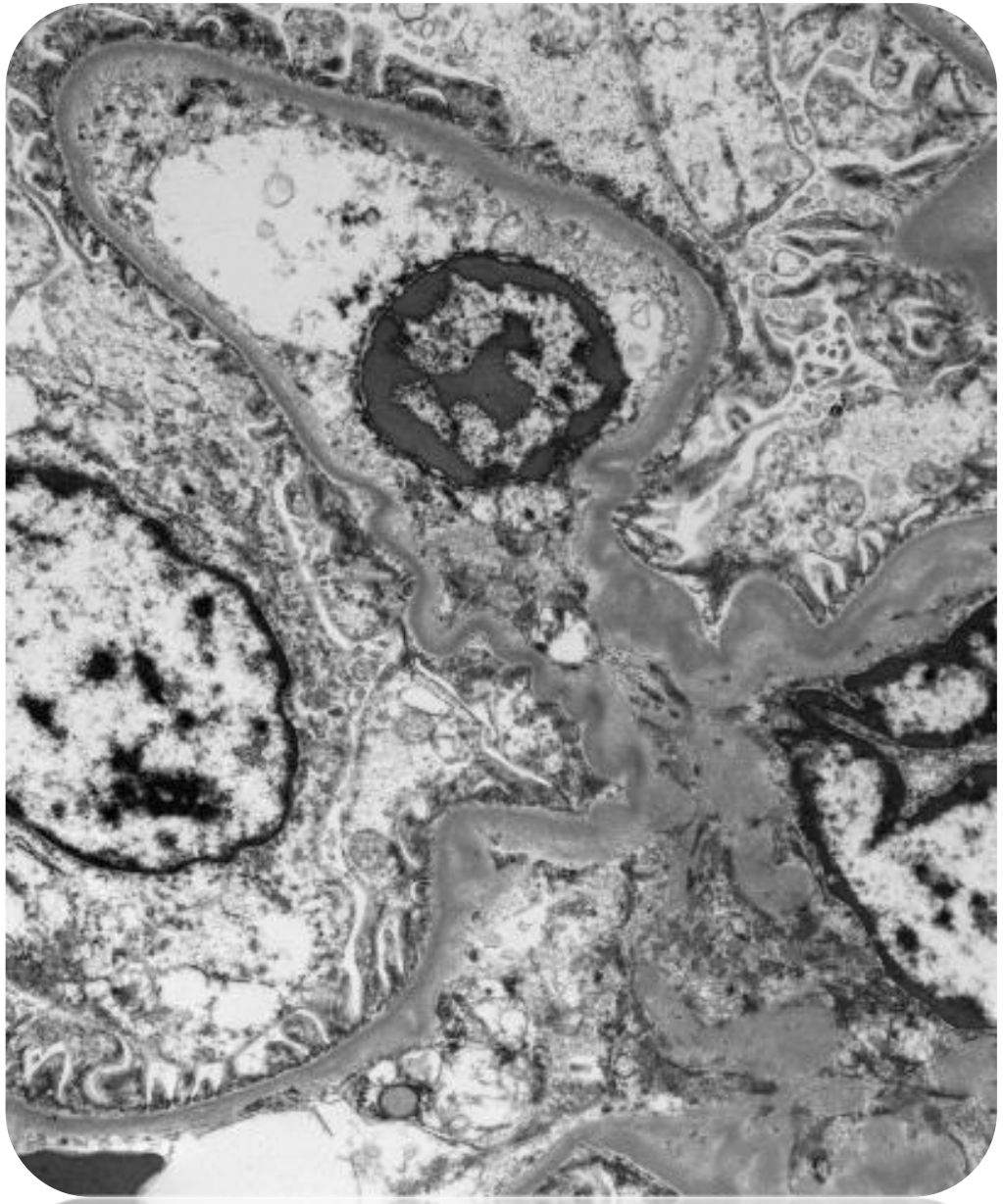
Glutaraldehyde at 5°C



Long term storage



Glutaraldehyde at 5°C



Long term storage

Long term storage, although not ideal, does preserve most of what may be relevant for diagnostic purposes, but we can see a definite lack of detail and slightly unusual appearance.

We would always recommend storage of tissue post processing, and long term storage as resin blocks, however, as long as the solutions are changed periodically to prevent fungal growth, long term wet tissue storage is an option.

Conclusions –

Unless fully known, the results of this pre analytical stage are fought with problems, issues, artefacts and complications we cannot possibly untangle. Receiving our biopsies in our ideal manner is always going to produce better results.
Being aware of these artefacts can make out job as screeners less problematic.

All EM reports are a combination of what has been found in other diagnostic tests, but sometimes these are not available, especially from referral centres. Another reason why renal EM is never a solitary diagnostic tool.

Bringing awareness of the impact of pre examination artefacts can help referral centres to understand the reason for our insistence that these biopsies are treated differently and the impact it can have if the protocols are not followed.
It helps us to avoid miss-reading our biopsies and make the right decisions when screening difficult to interpret images.