CHARACTERISING THE PROSTATE STEM CELL NICHE AND ITS ARCHITECTURE IN BENIGN PROSTATIC TISSUE

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ABSTRACT

Prostate cancer is the most common cancer among males in the UK with 1 in 8 men being diagnosed with the disease during their lifetimes. Despite its high prevalence and incidence, a lot about the disease process is still unknown. To understand the changes that occur in a malignant state, it is important to understand normal physiology and homeostatic mechanisms. It then becomes easier to pinpoint and understand what exactly goes wrong. Understanding the role of stem cells could also help in understanding castration-resistant prostate cancer as there could be cells that exhibit similar characteristics driving the tumour process at that point.

Key among the factors in maintaining a normal physiological state is the existence of prostate stem cells and prostate stem cell niches. There was a debate about the location of these cells – and whether they were basal or luminal. Previous work done also conclusively pointed towards a basal location although there was also evidence to say that luminal stem cells existed. Further work done in the lab previously, also confirmed these findings in addition to saying that these cells were clustered at the juxta-urethral prostatic ducts. There has also been research that has pointed to the existence of stem cells by discovering two cell types that did not fit into traditional classifications of prostate cells.

This study attempts to characterise the location of the stem cells and the stem cell niche within the larger context of prostate tissue. By using immunohistochemical methods to characterise each type of cell based on cell type-specific markers such as Prostate Specific Antigen and Uroplakin 1b, the aim is to paint a picture of the architecture of the stem cell niche and the surrounding microenvironment.

Some positive findings from this study could only add to the evidence that there exist certain areas of the prostate tissue which do not fall under traditional categorisations of prostate epithelium or urothelium. There also exist areas of overlap between prostate and urothelium which could point towards an important overlap in their origin stories – this needs to be studied further. However, for various reasons, the methods of study need to be optimised further for better results.

In conclusion, this project adds to evidence of a potential basal location for stem cells as well as talking about the various limitations with the methodologies used. In addition, there is also potential for future studies with regard to more structural as well as functional aspects of the niche including evaluating the role of stem-like cells in castration resistant prostate cancer.

LIST OF ABBREVIATIONS

34βE12- CK34βE12/keratin 903 (CK903)

3D – Three dimensional

AKR1C1 - Aldo-keto reductase family 1 member C1

AKR1C2 - Aldo-keto reductase family 1 member C2

ADT – Androgen Deprivation Therapy

AEC - 3-amino-9ethylcarbazole

AR – Androgen Receptor

AR6 – Antigen Retrieval Solution (pH6)

ASC – Adult Stem Cell

BCG – Bacillus Calmette Guerin

BPH – Benign Prostatic Hyperplasia

BRCA2 - BRCA2, DNA repair associated

BSA – Bovine Serum Albumin

CARN - Castration-Resistant Nkx3.1 expressing cells

CBC cells – Crypt base columnar cells

CK/KRT – Cytokeratin/Keratin

CSC – Cancer Stem Cell

DAB – Diaminobenzidine

DAPI - 4′,6-diamidino-2-phenylindole

DEG – Differentially Expressed Genes

DLK-1 – Delta-like Homologue-1

DMSO - Dimethyl sulfoxide

DPX - Distyrene Plasticiser Xylene

DRE – Digital Rectal Examination

ESC – Embryonic Stem Cell

FFPE – Formalin Fixed Paraffin Embedded

HRP – Horseradish Peroxidase

IgG – Immunoglobulin G

IF - Immunofluorescence

IHC - Immunohistochemistry

ISC – Intestinal Stem Cells

Ki-67 – Antigen Ki67

Lgr5 - Leucine-rich repeat-containing G-protein coupled receptor 5

MRI – Magnetic Resonance Imaging

mRNA – Messenger RNA

mtDNA – Mitochondrial DNA

NE cells – Neuroendocrine cells

NKX3.1 - Homeobox protein Nkx-3.1

OE- Other Epithelia (from paper by Henry et al.)

p63 – Tumour protein p63

PBS - Phosphate Buffered Saline

PSA – Prostate Specific Antigen

PSCA – Prostate Stem Cell Antigen

QuSAGE - Quantitative Set Analysis for Gene Expression

SCGB1A1 – Secretoglobulin A1

sc-RNA seq – Single Cell RNA Sequencing

SIMPLE – Sequential Immunoperoxidase Labelling and Erasing Method

TA cells – Transit Amplifying cells

TBS - Tris-Buffered Saline

TBST – TBS + Polysorbate 20 (also known as Tween 20) – TBS Tween

UGS – Urogenital Sinus

UPK 1b – Uroplakin-1b

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Chapter 1:Background

1.1. The Human Prostate

The human prostate is an important organ that is a part of the male genitourinary system. It is walnut shaped and sits below the urinary bladder and surrounds the male urethra. Its main function is to provide components of the seminal fluid with prostatic secretions forming about one-third of the same (Karthaus et al., 2014).

Development of the human prostate is part of a two-pronged approach to male sexual differentiation. The first part involves the regression of the Mullerian duct system – a system that develops at around 6 weeks of gestation in all foetuses and eventually form the Mullerian tubercle at around 8 weeks of gestation. This regression takes place due to the presence of the anti-Mullerian hormone expressed in testicular Sertoli cells and the stabilisation by androgens of the Wolffian ducts – which have developed 25-30 days after conception and act as excretory ducts till the formation of a definitive kidney, after which they become incorporated into the genital system.

The second part of male sexual differentiation involves the influence of androgens produced by the foetal testis – specifically the Leydig cells in the testis. The process involves the formation of the vasa efferentia, the epididymal ducts and the vas deferens. It also forms the prostate and the prostatic utricle in addition to other processes such as the closure of the scrotal-labial folds, masculinisation of the urogenital sinus (UGS) and the external genitalia being formed.

The rudimentary prostate appear in 50mm human embryos as epithelial buds growing laterally from the walls of the UGS at the Mullerian tubercle. Under local mesenchymal control, the buds form solid branching cords which start to develop a lumen. Eventually they give rise to a network of tubules and alveoli in a process that is complete by birth. As the lumen forms, some of the apical cells become structurally polarized and appear to start some secretory activity. The organ develops a stroma containing a large proportion of smooth muscle while the ducts and acini are lined with a layer of flat basal epithelium and a luminal layer of tall columnar secretory epithelium.

Development of the rodent prostate as established by many studies, mirrors the human prostate embryogenesis but with an accelerated time scale. For example, the development of the prostate begins with prostatic buds from the foetal urogenital sinus. This begins at 17 days of gestation in the mouse, 19 days in the rat and 10 weeks in the human foetus.

McNeal et al in 1969 elaborated upon a zonal architecture of the fully developed human prostate which was in sharp contrast to that of the mouse prostate. The human prostate could be divided into three zones – the central zone, the peripheral zone and the transitional zone in between the former two. These were also surrounded by an anterior fibromuscular stroma (McNeal, 1969). The central zone is a wedge of glandular tissue which

constitutes most of the base of the prostate and surrounds the ejaculatory ducts. The peripheral zone surrounded most of the central zone and extended caudally to partially surround the distal portion of the urethra.

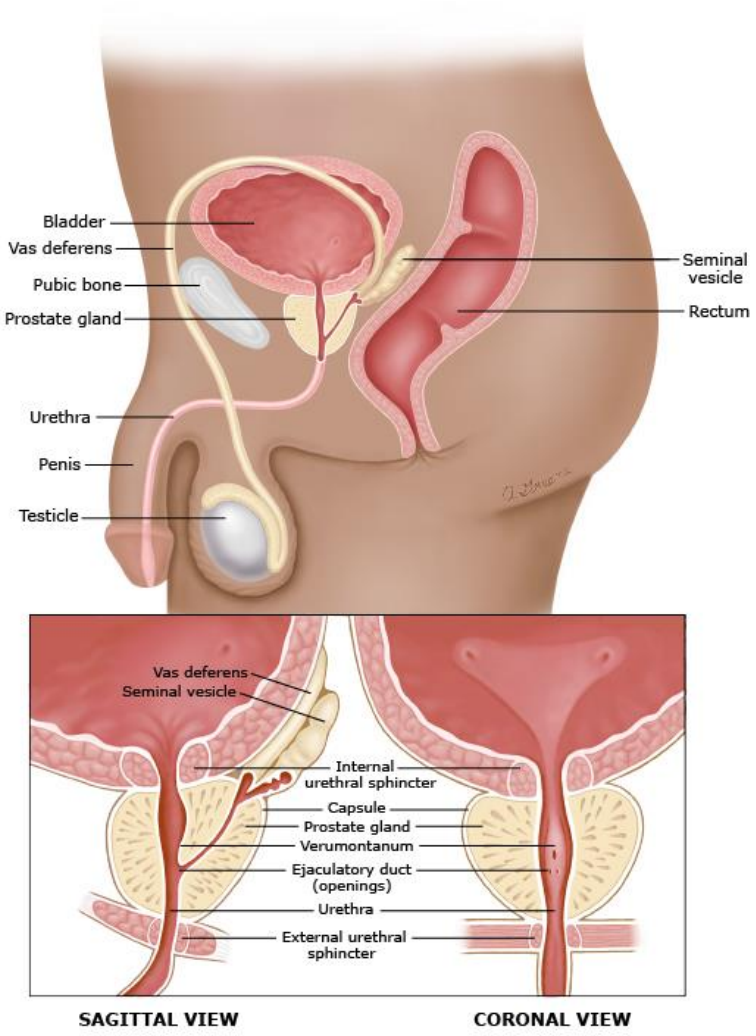


Figure 1.1 – Location of the prostate within the human body with both the sagittal and coronal views of the prostate gland to show its anatomical relations.

Prostatic tissue consists of multiple glandular subunits which drain into the prostatic urethra proximally. These subunits consist of ducts which are lined by prostatic

epithelium. The epithelium consists of basal cells, luminal cells and neuroendocrine cells. The luminal cells and basal cells are arranged in pseudostratified arrangement with interspersed neuroendocrine cells. The rare neuroendocrine cells interspersed within the basal layer are believed to aid the growth of luminal cells through paracrine signalling (Shen et al., 2010).

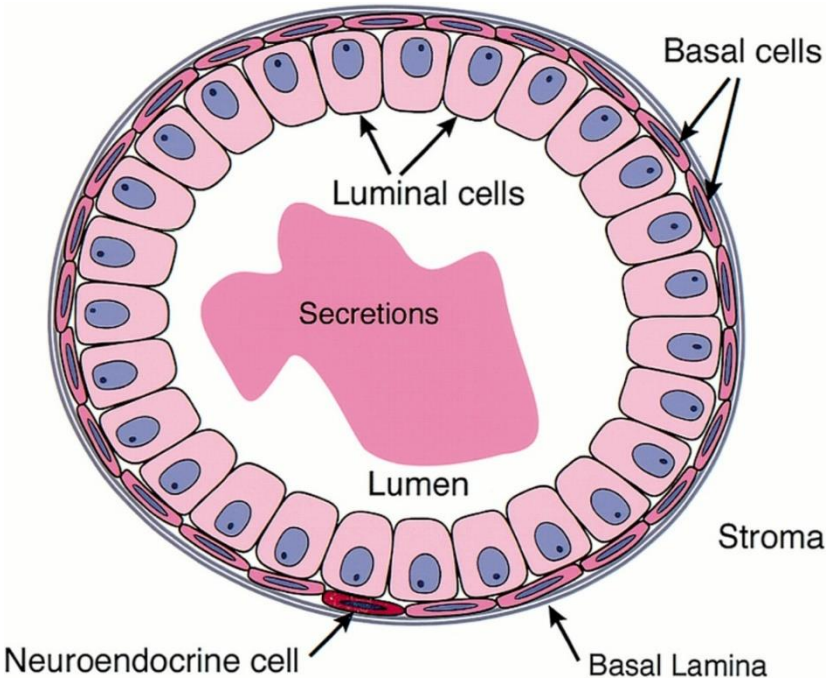


Figure 1.2 - ***Prostate anatomy and morphology*** *-Epithelial cell types found in the human prostatic gland. Adapted from Abate-Shen et al. 2000*

On the other hand, rodent prostate is not one compact anatomical structure. Instead it is a collection of four distinct lobular structures, each with its own lobe-specific branching morphogenesis. The four lobes are named ventral, dorsal, lateral and anterior. The ventral lobes in both rat and mice are located below the urinary bladder on the ventral aspect of the urethra. The lateral lobes lie just below the coagulating glands and the seminal vesicles. The dorsal lobes are both inferior and posterior to the urinary bladder and the anterior lobes (also known as the coagulating glands) lie adjacent to the seminal vesicles.

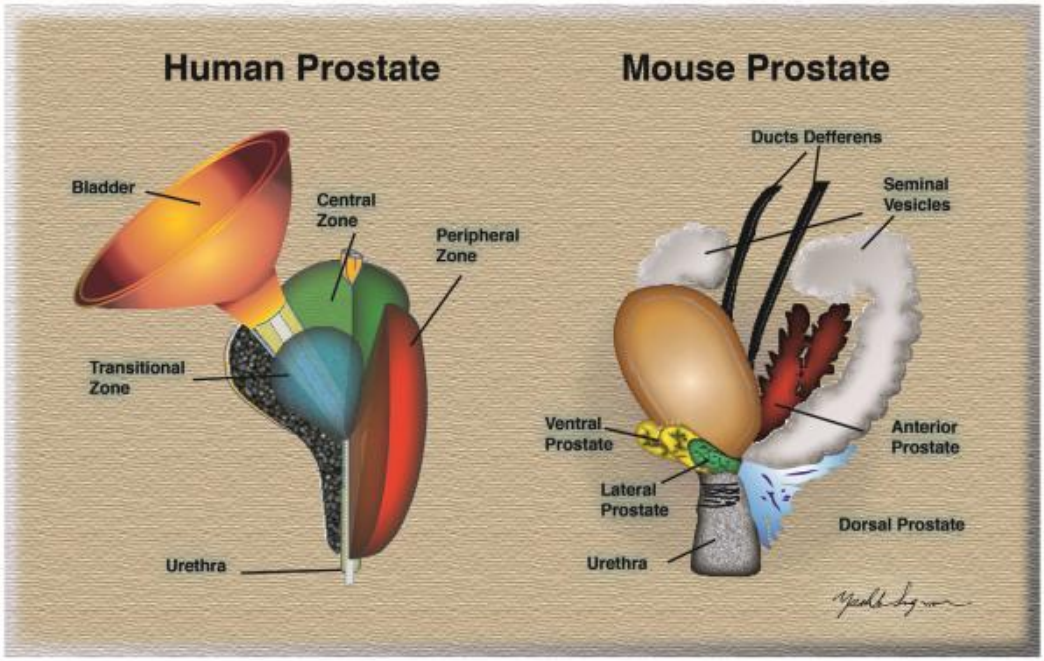


Figure 1.3 - A comparison of the human prostate versus the mouse prostate showing the zonal architecture in human prostate versus the lobular organisation of the mouse prostate. Adapted from Sugimura Y, Cunha GR, Donjacour AA. Morphogenesis of ductal networks in the mouse prostate. Biol Reprod 1986;34:961-71; with permission

While lobe versus zone homology has been put forth by many studies with respect to rodent versus human prostate, the Bar Harbor Consensus meeting in 2001 concluded that “there is no existing supporting evidence for a direct relationship between the specific mouse prostate lobes and the specific zones in the human prostate”.

Diseases in the prostate can be benign or malignant and both are common. Symptomatic Benign Prostatic Hyperplasia (BPH) affects more than 40% of men over 50 years of age. Malignancy of the prostate is also one of the most common diseases among the male population and is discussed further in detail below.

1.2. Prostate Cancer

Prostate cancer is the most common cancer in the UK among males with the exception of non-melanoma skin cancer. It is the most commonly diagnosed cancer in the UK. More than 47,500 men are diagnosed every year with prostate cancer (129 men everyday) and roughly 11,500 die from the disease every year. 1 in 8 men will be diagnosed with prostate cancer in their lifetime and around 400,000 men are living with or after prostate cancer (Cancer Research UK, 2017).

Despite the severity and magnitude of the disease, relatively little is known about the cause or factors that influence the development of the disease. To this day, age,

ethnicity and family history of prostate cancer remain the only major established risk factors for the disease. (Cancer Research UK, 2017)

However there are certain molecular tests that have come to the fore in recent years which have tried to improve the predictability of disease prognosis – such as The Decipher® Prostate Cancer Test (GenomeDx Biosciences, San Diego, CA) (Dalela et al., 2016). Moreover, models such as the Stockholm-3 model for prostate cancer detection have also been shown to be more efficient even at screening diseases than traditional Prostate-Specific Antigen (PSA) based screening (Strom et al., 2018; Eklund et al., 2018). With time, the efficiency of both screening tests and prognostic tests should only improve leading to better overall disease outcomes.

Prostate biomarkers that are also used in addition to PSA for screening of diseases include pro2PSA, Prostate Cancer Gene 3 (PCA3), urinary mRNA levels of HOXC6 and DLX1 proteins. These are often used in conjunction with PSA levels as well as Gleason scoring for prostate cancer in order to determine various aspects of the disease including extent of spread at the time of investigation as well as potential prognosis of the disease.

Gene panels are also used such as in Oncotype DX – Genomic Prostate Score (GPS) to look at prognosis of the disease in patients who have biopsy proven prostate cancer considering active surveillance. For patients with advanced disease and to help decide on systemic therapies, AR-V7 is a truncated Androgen Receptor (AR) circulating tumour cells activated independent of androgen binding. The results of AR-V7 testing could determine the approach to further therapy and whether the patient would benefit from non-androgen therapies.

1.3. Risk Factors

1.3.1 Age

The estimated risk for developing prostate cancer is 1 in 6 in males born after 1960 in the UK. The development of prostate cancer is linked to increasing age in males and its incidence is greatest in elderly males. Between 2012 and 2014, those over the age of 70 accounted for more than half of the new cases that were diagnosed. The number of new cases diagnosed among the population of men under 50 years of age was less than 1%. Between 2014 and 2035, the number of cases of prostate cancer are

projected to rise by 12%. The increase can partially be attributed to an ageing population. (Cancer Research UK, 2017)

1.3.2. Ethnicity

Ethnicity is also a major factor in the development of prostate cancer. Age standardised incidence rates of prostate cancer are significantly higher for black males in England as compared to white males - ranging from 120.8 to 247.9 per 100,000 for the former versus 96.0 to 99.9 per 100,000 for the latter. The incidence is also significantly lower (28.7 to 60.6 per 100,000) for Asian males. The lifetime risk of being diagnosed with prostate cancer is 13.2-15.0% for White males, while in Black males it is significantly higher (23.5-37.2%), and in Asian males it is significantly lower (6.3-10.5%). Similar findings were established in a separate study in the United States but factors around diagnosis do not account for the difference in incidence based on ethnicity (Lloyd et al., 2015; National Cancer Intelligence Network and Cancer Research UK, 2009; Ben-Shlomo et al, 2008; Metcalfe et al., 2008).

1.3.3. Family History

Family history is also a significant player in the development of prostate cancer with inherited factors explaining around 5-9% of prostate cancers. Prostate cancer risk is 2.9-3.3 times higher in men whose brothers have had the disease and 2.1-2.4 times higher in men whose fathers have had the disease. Prostate cancer risk is 1.9 times higher in men with a second-degree relative (grandfather or uncle, nephew, or half-sibling) who has/had the disease. The existence of a genetic link is supported by the fact that prostate cancer risk in a person is not associated with risk in the adoptive parent. Approximately 10% of people have true hereditary prostate cancer (patients with at least 3 affected relatives) and they make up for more than 40% of early-onset prostate carcinoma cases (diagnosed before the age off 55 years). Predisposition due to genetic mutations such as that in *BRCA2* are rare and account for 1% of early onset cases with a very aggressive presentation. (Bruner et al., 2003; Johns et al., 2003; Kicinski et al., 2011; Cancer risks in *BRCA2* mutation carriers, 1999)

1.4. Characteristics of prostate cancer

Most prostate cancers are adenocarcinomas with a luminal phenotype. Most tumours present within the peripheral zone (75%) while the central zone accounts for the least at 5%. The transitional zone accounts for the remaining 20% (McNeal et al., 1988).

One of the main features of prostate cancer is the multifocal nature of the disease which consists of one dominant tumour – the index tumour – and multiple smaller tumours. (Bostwick et al., 1998; Arora et al., 2004; Ruijter et al., 1996; Wise et al., 2002)

1.5. Metastasis

Metastasis is also a common problem that occurs with the disease. While in theory, cancer cells from the primary tumour can migrate to any other part of the body, the most common metastatic sites for prostate cancer are the lymph nodes and the skeletal system. Spinal metastasis is a common sequela of the disease when it turns metastatic. The four most common sites of metastasis are bone (especially the lumbar spine), lymph nodes, lungs and liver. The spread of prostate cancer can be either lymphatic or haematogenous in nature. Other less common sites of metastasis include the adrenal glands, brain, breasts (in females), eyes, kidneys, muscles, pancreas, salivary glands, and spleen.

1.6. Diagnosis

The main methods to diagnose prostate cancer are through Digital Rectal Examination (DRE), serum prostate specific antigen (PSA) levels and using ultrasound guided biopsies. Magnetic resonance imaging (MRI) is used to visualise the prostate and helps target biopsies. Confirmatory diagnosis of prostate cancer is dependent on needle biopsy results from the prostate under transrectal ultrasound guidance. (Heidenreich et al., 2014)

1.7.Treatment

The main modality of treatment for localised prostate cancer consists of either radical prostatectomy, irradiation by external beam radiotherapy, or brachytherapy (low dose radiation therapy from radioactive seeds inserted into the prostate) (Shen et al., 2010). The main challenge lies in detecting indolent disease which may be harmless and may not require treatment from disease likely to cause clinical symptoms, metastasis and death. Right now, the norm is to treat men with indolent disease by active surveillance which can help avoid or delay radical surgery or radiotherapy. In case of locally advanced prostate cancer, a combination of local treatment and systemic treatment is used and is found to be effective. Long term Androgen Deprivation Therapy (ADT) combined with radiotherapy has proven through randomised controlled trials to be more effective than either one alone (NICE guidelines, 2019).

ADT is the mainstay for advanced metastatic stage of prostate cancer, where they limit prostate tumour growth as the tumour cells typically depend upon stimulation of androgen receptor (AR) activation for continued proliferation. Unfortunately in the vast majority of men these tumours progress to castrate resistant tumours after ADT (Henshall et al., 2001). One possible explanation for this feature of late stage prostate disease would be the existence of a subpopulation of tumour cells that are castrate resistant and capable of repopulating the tumour following ADT. It has been suggested these cells are cancer stem cells (CSCs), which might derive from adult stem cells within the prostate. (Reya et al., 2001)

In the metastatic setting, the consensus is to manage the primary tumour with radiotherapy. Whether radical prostatectomy would be of benefit in these patients is unclear and the subject of many studies that are ongoing. Treatment of the metastases would depend on the nature of metastases – whether the disease is oligometastatic (having a limited number of synchronous or metachronous metastases in bones or lymph nodes but not visceral organs) or widely metastatic. The treatment modalities vary slightly but the use of radium-223 treatment for metastatic disease, the use of abariterone along with glucocorticoids such as prednisolone and treatment with cabazitaxel in addition to the treatment of primary disease with ADT and/or radiotherapy as mentioned above depending on hormone sensitivity is the recommended strategy.

1.8. The Stem Cell

Stem cells have been described as having the ability to self-renew and differentiate into mature cells of a specific tissue type. Stem cell existence has been talked about and elaborated through various studies on different human organs including hair follicles, skin, intestinal tissue and bone marrow. The presence and activity of the stem cells within these organs allow for the maintenance and regeneration of tissue that need to be replaced due to injury or ageing for example.

Stem cells have unique characteristics that often differ from the other types of cells that make up the organ. By their very nature, stem cells are quiescent – only engaging in activity when called upon by physiological circumstances. They also are very small in number and represent a very small proportion of the total number of cells. They are also located in specific, highly specialised and confined locations as well called as “niches” (Takao et al., 2008).

Ray Schofield suggested in 1978 that the stemness of these cells came from not just their ability to self-renew but also was a product of their interaction with their micro-environment. It is this interaction plus the inherent characteristics of a stem cell that both contribute to a niche.

There are different types of stem cells – including embryonic stem cells (ESCs) and adult stem cells (ASCs). The main difference between the two is that ESCs have the characteristic of pluripotency – meaning that these stem cells can differentiate into any cell type depending on the conditions it is subjected to. The ASCs on the other hand, have restricted lineages where they are confined to becoming a single type of mature cell. It is these types of stem cells that maintain the prostate (Rossi et al., 2008)

1.9. Stem Cells in the Prostate

1.9.1 Studies on Murine Prostate

Just like in many other cases, the evidence for the existence of stem cells within the prostate came about from studies done on murine prostatic epithelium (rat). As mentioned above the prostatic epithelium contains three types of cells.

i. Cells that line the ductal lumens within the prostate – known as luminal cells
ii. Cells that are adjacent to the basal membrane and are underneath the luminal cells – called basal cells
iii. Secretory cells expressing neuropeptides, distinct from basal and luminal cells and very rare in occurrence – called neuroendocrine cells.

Studies by English et al. and Evans et al. were the first studies that demonstrated that androgen deprivation leads to the regression of adult prostate. They also showed that the apoptotic process that ensued affected luminal cells more than basal cells.

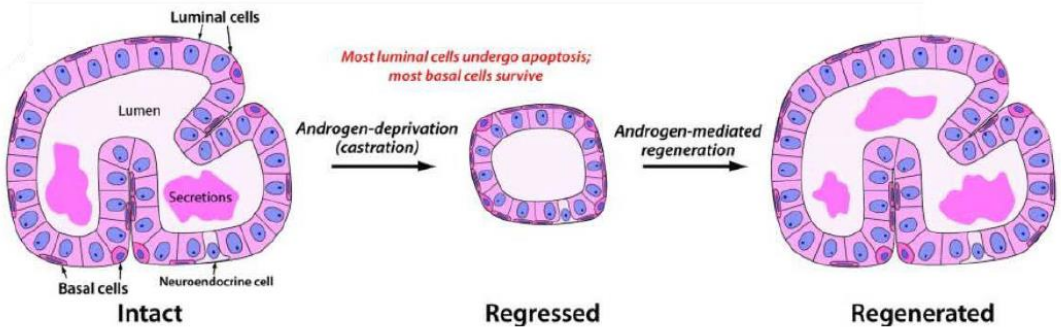


Figure 1.4: Illustration showing androgen deprivation and restoration cycle. Under a state of androgen deprivation, basal cells survive while luminal cells undergo apoptosis in the murine prostate. And when

androgen is restored, it enables the tissue to regenerate back to a normal prostate with luminal, basal and neuroendocrine cells. Adapted from Wang et al, 2009.

Studies involving the transplantation of embryonic murine p63+ urogenital sinus (UGS) into immunodeficient mice then contributed to being another key piece of evidence in the study of the human prostate stem cells. Tumor protein 63 (p63) is a transcription factor of the p53 gene family involved in differentiation of several tissues including squamous epithelium. Within the prostate, p63 is a basal cell marker that is present from the embryological stage in both humans and rodents. The p63+ UGS did not differentiate into basal cells but did differentiate into luminal and neuroendocrine cells – indicating that basal cells are not needed for prostate-like tissue generation in transplantation assays. (Evans et al.,1987). Signoretti et al. then conducted chimeric studies on rats between the wild type and p63 knockout mice proving that only p63+ mice could develop prostate epithelium comprising of basal and luminal cells. This implied that all prostate cells arise from p63+ progenitor cells during normal murine embryonic development. All of these studies *in toto* pointed towards the existence of castrate-resistant adult prostate stem cells within the prostatic epithelium.

1.9.2. Location of the Prostate Stem Cells

The question then turned towards the location of the prostate stem cell. It was seen that p63- mice cannot develop a prostate and p63 is expressed in basal cells only – hence leading to the conclusion that basal p63+ progenitor cells were the ones which gave rise to both basal and luminal cells. Hence the basal compartment – which was positive for p63 – became the focus of further research into the location of the stem cell. Further supporting this idea was the study done by Leong et al. where a single adult basal cell could regenerate the prostate tissue on transplantation supporting the existence of adult multipotent prostate stem cells within the basal region

A small population of basal cells in both human and mice prostate do show the characteristic of multipotency when looked at with functional prostate regeneration assays but these conditions do not mimic physiological states and hence their data must be looked at keeping that in mind. (Leong et al., 2008; Burger et al., 2005; Goldstein et al., 2010; Lawson et al., 2007; Xin et al., 2003)

1.9.3. Lineage Tracing Studies

Precursory to the advent of lineage tracing studies, their importance was demonstrated by Molyneux et al. (2010), when they showed that results obtained from experiments

looking to determine cell of origin but without functional lineage tracing would be unreliable. Other problems associated with methods used in determining the solution to this particular issue (including transplantation of UGS and in vitro methods of primary prostate culture) include the preference for basal cell growth over luminal cells (Cunha et al., 1973; Garraway et al., 2010). This meant that knowledge about the luminal cell and its possible role relatively unclear. The existence of a murine luminal stem cell which could potentially account for the origin of prostate cancer was demonstrated by Wang et al. (2009) in a castrate resistant model – which, while important, is not something we expect to see in normal physiological conditions.

The emergence of lineage tracing studies has proven to be a blessing for the study of stem cells in the prostate. Lineage tracing, in simple terms, is the identification of all the progeny of a single cell. In lineage tracing studies, a single cell is marked in such a way that the mark is transmitted to subsequent generations of cells leading to a set of labelled clones. The lineage tracer must have certain properties for it to be effective – namely, that it must not change the properties of the marked cell, its neighbours or its progeny, it must be passed on to all the progeny of the founder cell and it must be sustained over a period of time. Various methods of lineage tracing exist including direct observation (for example using time lapse microscopy), labelling cells with radioactive dyes and radioactive tracers, introduction of genetic markers through transfection or viral transduction, direct transplantation of cells and tissues from one embryo to a different host, creation of genetic mosaics (for example with chimeric mice), and cell marking by genetic recombination. Increasingly, studies of lineage tracing with multicolour reporters allowing the observation of multiple cell lines are also gaining importance. While each method has its own advantages and disadvantages depending on the final objective, the underlying principle of observing the fate of cell lines remain the same.

Lineage tracing studies by Wang et al. demonstrated that luminal cells expressed Nkx3.1 (a prostate epithelial protein that is coded for by the *Nkx3.1* gene located on chromosome 8p – it is a prostate specific tumour suppressor gene) for the most part while only a handful of basal cells were seen to be expressing the same. Upon castration however, the results were that Nkx3.1 expression was massively reduced from the luminal cells. The cells which continued to express Nkx3.1 were termed as Castration-Resistant Nkx3.1 expressing cells – CARNs. These cells were then shown

to generate both basal and luminal cells but once again, due to the effect of castration it cannot be said to accurately reflect physiological homeostasis.

Further lineage studies were done by two groups (Ousset et al. (2012); Choi et al., (2012)) to potentially examine the stemness of both basal and luminal cells. Choi's group suggested the existence of self-sustaining, independent populations of unipotent luminal and basal cells within the prostate. The experiment involved labelling basal cells with Cytokeratin-14 (cytokeratins are proteins that are found in the intracytoplasmic cytoskeleton of epithelial tissue) and luminal cells with CK-8 (both specific to the tissue they mark) and the results were that neither was able to differentiate into the other lineage. Ousset's group used the same markers as Choi (CK14 and CK5 for basal cells and CK8 for luminal cells) and looked at both embryonic and adult prostate tissue. During embryonic development CK14 positive basal cells expanded in number and differentiated into both CK5 positive basal and CK8 positive luminal cells. Lineage tracing studies done on the CK5 positive cells showed a high proportion of basal cells versus luminal cells and the CK8 positive cells displayed no evidence of differentiation into basal cells whilst also showing a stable number of luminal cells. Interestingly, one other type of cell was also shown by the studies – the "intermediate" adult cells. These were shown to be basal in nature but with the expression of both CK5 and CK8 – markers for luminal cells. This led to the conclusion that while adult prostate regeneration showed distinct basal and luminal stem cells that mediated the process, the same could not be said of the embryonic stage where multipotent basal progenitor cells drive the process of prostate generation.

Thus the natural corollary was that there existed unipotent luminal progenitors in both the embryonic stage and the adult stage of the murine prostate while basal progenitors progress from being multipotent to being unipotent as they develop from embryo to adult. The key here though is that there is a small population of cells that could potentially still be multipotent even in adult murine prostate.

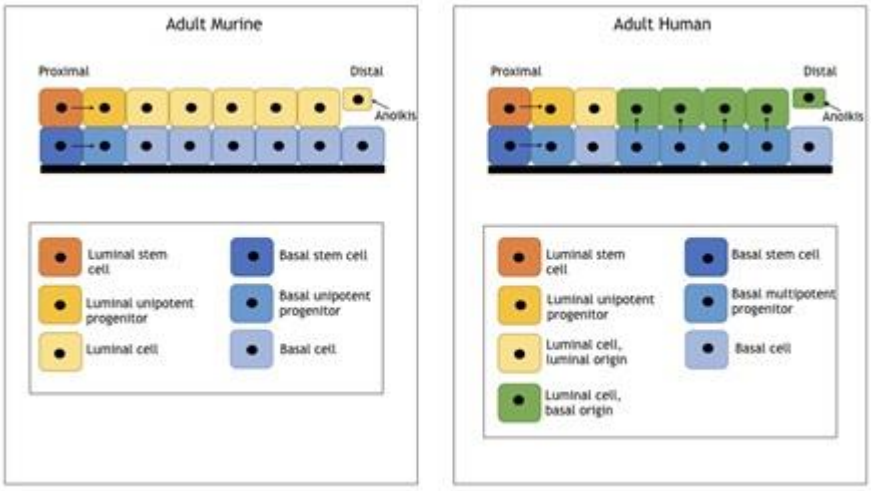


Figure 1.5: Diagram demonstrating adult murine and human prostatic epithelium. From Human Prostate Stem Cells and Their Niche - A Comprehensive Review (Subramanian et al., 2019)

1.9.4. Studies on Human Prostate and Associated Problems

Karthaus et al. also argued in their work that the physiological conditions of prostate tissue is not replicated in in vitro culture systems. UGS transplantation and primary prostate cell culture for example do not contain androgen receptors (AR) at physiological levels and the tissues generated do not resemble in vivo prostate tissue. Karthaus' group derived their work from work done on the gastrointestinal tract by Sato et al. (2009) and Sato et al. (2011) – producing R-spondin1 based adult prostate organoids from both murine and human prostates. The basal cells within the human model expressed p63 and CK5 while the luminal cells expressed CK8 and AR – in keeping with their nature. The two different types of cells were then separated and cultured to form organoids. Basal cell (CK5 positive) derived organoids expressed mostly CK5 with CK8 positive luminal cells found around some sporadic luminal formations. It also showed patchy AR expression. Organoids derived from luminal cells immediately formed lumens. Most of the cells within the organoid expressed CK8 and AR with a small minority expressing basal marker CK5 – these were basal cells. This leads to the natural conclusion that both basal and luminal cells in a controlled, organoid model have the ability to generate the other type of cell i.e. they display bipotency.

However, given that all these studies were performed on murine prostate models, it was difficult to see how much of this data could be reliably extrapolated to human prostate. The advent of mitochondrial DNA (mtDNA) marks – linked to ageing – that could be used for lineage tracing would change that by allowing us to study the fate of cells within a human setting. The mtDNA mutations lead to deficits in the respiratory

chain that can be identified using Cyclo-oxygenase activity. (Blackwood et al. (2011); Gaisa et al., (2011))

Work done by Moad et al. (2017) which used lineage tracing methods of human prostatectomy specimens and subsequent in vitro organoid culture eventually demonstrated the multipotency of basal stem cells and unipotency of luminal stem cells. This provides us with a clear evidence of the existence of multipotent basal stem cells but also gives us conflicting evidence with regard to multipotent and unipotent behaviour in the luminal cells.

1.10. Stem Cell Niche Within The Prostate

Stem cells in any organ do not exist on their own. Adult stem cells are located in specific, confined locations within the organ often with highly specialised and unique microenvironments which facilitate the maintenance of tissue homeostasis between the processes of cellular quiescence and cellular activity.

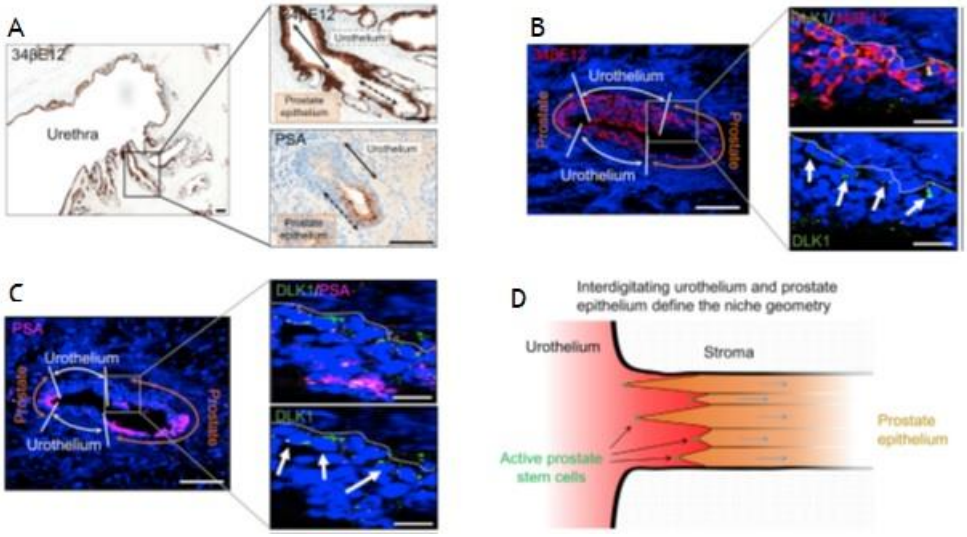


Figure 1.6 – Adapted from Multipotent Basal Stem Cells, Maintained in Localized Proximal Niches, Support Directed Long-Ranging Epithelial Flows in Human Prostates, Moad et al (2017)
(A) The juxta-urethral prostate ducts show variable encroachment of urothelium along the longitudinal axis, marked by 34betaE12 (expressed in all layers of
urothelium but basal only in the prostate epithelium) and, in the next sequential slide in the z-plane, PSA (prostate luminal cells only). Scale bars, 100 mm.
(B and C) Two consecutive sections of the same gland illustrate the urothelial-prostate epithelium boundary, described by (B) 34betaE12 and (G) PSA immunofluorescence
in the radial axis, and demonstrate an interdigitating pattern on which DLK1+ve basal prostate stem cells are positioned. Scale bars, 20 mm.
(D) Sketch of the spatial arrangement of cells types at the niche (cross-section along the longitudinal axis of the proximal truck). Prostate stem cells are localized in
between urethral and prostatic epithelial interdigitation, giving rise to transiently expanding clonal streams.

Work done by Moad et al. (2017) is comprehensive in its description of the prostate stem cell niche. Lineage tracing demonstrated the bipotency of basal stem cells and

the unipotency of luminal stem cells. Three dimension glandular reconstructions with proliferation kinetics and functional assays of differentiation showed some important results.

1. Stem cells gave rise to migratory streams of cells – continuous in nature – originating from individual stem cells from the proximal ductal epithelium (basal layer) to the distal ductal epithelium.
2. Proximally located luminal cells had their own unipotent cells. These gave rise to short clonal expansions of luminal-only cells from the proximal part of the duct.
3. There exists an interdigitation of urothelium with the prostatic epithelium into the prostatic ducts. Basal progenitors lie in the interface between urothelium and the prostatic epithelium within these separating interdigitations.

Findings of a proximal location of the stem cell niche were also supported by work on murine prostate tissue by Tsujimura et al. (2002), who demonstrated that murine prostate stem cells come from the proximal portion of the duct.

All of these point towards a basal layer in the stem cell niche as the origin for human prostatic stem cells. They are nested within the interdigitations and exhibit unidirectional flow distally into the ducts where they demonstrate both multipotency and unipotency. There also exists a separate contingent of luminal stem cells responsible for maintaining the luminal epithelium in the proximal part of the duct.

1.11. Prostate Club and Hillock Cells

For a long time, our understanding of cells within the prostate have been shaped by the definition of the cell types based on surface antigens, gene expression, shape and relative position (towards the basement membrane versus the lumen). This has led to the identification of three different epithelial cell types – basal, luminal and neuroendocrine cells. It has been seen that basal cells express CK5 and p63, luminal cells express CK8. NE epithelia show the expression of chromogranin A. A comprehensive list of markers that are present within prostate tissue, urothelial tissue and other related cells are shown in Table 2 below. However, while these were the tools used to conventionally label basal, luminal and NE cells, the purity of their labelling was never accounted for.

Table 1 – List of potential targets to be marked out with IHC and the types of cells they mark out along with the area of the cell (nuclear/cytoplasmic) that the mark out

Candidate Marker	Location
34BetaE12	Basal, cytoplasmic
p63	Basal, nucleus
Androgen Receptor	Luminal, nuclear
Prostate Specific Antigen	Luminal, cytoplasmic
CK8+18	Luminal, cytoplasmic
NKX3.1	Luminal, nuclear
Ki-67	Proliferative cells, nuclear
DLK-1	Prostate Epithelial Stem Cells
Uroplakin	Urothelium

Work done by Henry et al. (2018) involved single cell RNA sequencing (scRNA-seq) on close to 98,000 cells from five young adult human prostates. To begin with, flow cytometry (FACS) was done to bulk sequence human prostate cells and it suggested the existence of multiple phenotypes of cells. Following this, single cell RNA sequencing established that there existed multiple different cell types within the broad framework of epithelial and stromal cells. Their work identified five different epithelial cell types as opposed to three and in addition, established cell types could now be identified by previously unknown markers. One finding that is crucial to note is that regardless of which basal markers were used, there existed a group of cells that consistently showed a double-negative epithelial gate for certain basal cells which has never been characterised before.

The sequencing helped identify subclusters of epithelial cells with data from bulk-sorted prostate epithelial cell transcriptomes. They revealed two clusters which correlated well with the double negative epithelial gate mentioned above in addition to

another cluster which correlated well to bulk-sequenced basal epithelia. The two clusters (Clusters 1 and 6 in the data shown below from their figures) were tentatively named OE1 and OE2 (OE standing for ‘Other Epithelia’).

OE1 and OE2 were positive for SCGB1A1 (Secretoglobulin 1A1) and cytokeratin 13 respectively – which was confirmed by Differentially Expressed Gene sets (DEGs). Of interest is the fact that secretoglobulins are found to be highly expressed by club cells within the respiratory tract. There is previous evidence that SCGB1A1 has been seen in the human prostate as well. QuSAGE data comparing human prostate epithelial scRNA sequencing with that of scRNA sequencing data from mouse lung epithelia demonstrates a strong link between prostate epithelia positive for CK13 and CK5 and club cells (CK5 positive) in the lung along with hillock cells in the lung (which are CK13 positive).

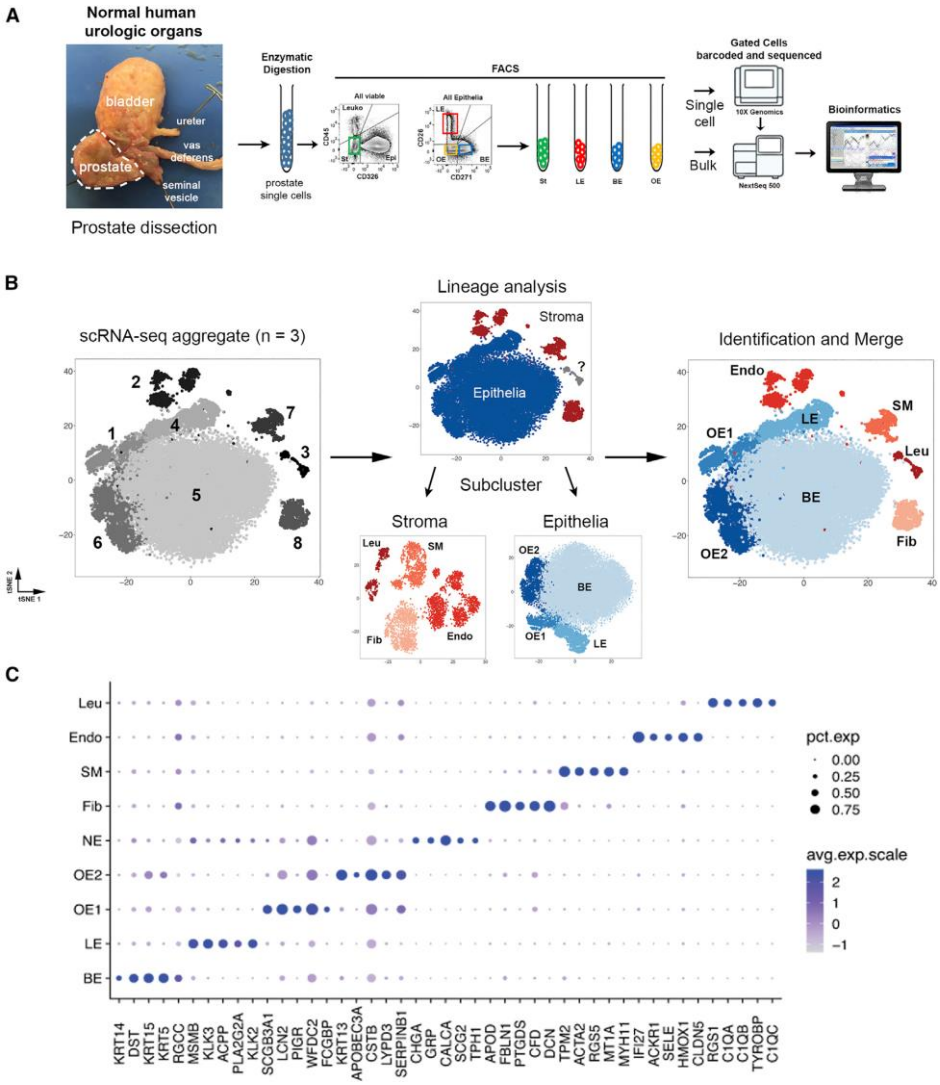


Figure 1.7. Identification of Human Prostate Cell Clusters with Bulk and Single-Cell RNA Sequencing (A) Schematic of human tissue collection and processing for bulk and single-cell RNA sequencing.

(B) Aggregated single-cell RNA sequencing (scRNA-seq) data from three organ donor prostate specimens with subclustering into stroma, epithelia, and unknown

lineages based on correlation with bulk sequencing data (Figure S2). Clusters were identified and re-merged.

(C) Dot plot of cluster-specific genes after in silico removal of stressed cells and supervised identification of neuroendocrine epithelia.

Club cells (previously named Clara Cells) are nonciliated, nonmucous, secretory cells in respiratory epithelium. Club cells are morphologically columnar to cuboidal with a distinctive dome-shaped luminal surface with small periodic acid-Schiff (PAS)-positive secretory granules. The primary functions of club cells are: (1) to provide secretory surfactants (surfactant proteins A, B, and D) and other specific proteins that contribute to the airway epithelial lining fluid; (2) to serve as progenitor cells for ciliated and secretory epithelial cells; and (3) to metabolize xenobiotic compounds through cytochromes P450-dependent mixed-function oxygenases.

Hillock cells in the lung on the other hand are CK13 positive, transitional cells which express regulators of cellular adhesion and squamous epithelial differentiation and genes associated with immunomodulation and asthma. They are high turnover cells which are important in injury response.

Functional analysis of each cell type were undertaken with each of these epithelial subclusters. Transcriptomes from these were used to run QuSAGE against previously established databases of C2 curated gene sets. From this, it was seen that OE1 and OE2 and immunomodulatory pathways showed a strong correlation – indicating that they could potentially have a role to play in that system.

In-situ immunofluorescence labelling for each cell type showed that central and transitional zones of the prostate are rich in both OE1 and OE2 (see figure) but luminal epithelia are low in number. The prostatic urethra, the collecting ducts and the central zone surrounding the ejaculatory ducts saw an abundance of CK13+ hillock cells. SCGAB1A1+ club cells meanwhile populate the prostatic urethra as well as the collecting ducts but are rare in the prostate itself.

Two characteristics that cement the belief that these could potentially be related to stem cells and stem behaviour within the prostate are

1. Flow cytometry data to quantify Prostate Stem Cell Antigen (PSCA) showed that PSCA+ other epithelia (OE) are enriched as a percentage of epithelia in the central and transition zones.

2. Pseudotime analysis used to understand the relationships between the four cell types (basal, luminal, club and hillock) shows that basal cells eventually give rise to the other three cell types – somewhat like in the lung where CK5 positive basal epithelial cells are multipotent and give rise to all differentiated cell types but the club and hillock cells of the lung are less broad in terms of their differentiation abilities.

It is currently unclear if the prostate hillock cells populate the foetal urogenital sinus (UGS) and hence their role in the embryonic development of the prostate is still a question that needs to be answered. However, CK13 positive cells are seen to be enriched in localised prostate cancer and stem-like cells that display both branching morphogenesis and a resistance to androgens. This is further substantiated by the fact that the two main genes of the CK13 positive cell type are both involved in the androgen metabolism pathway (the genes being AKR1C1 and AKR1C2) – which could also contribute to the development of castration-resistant prostate cancer. This suggests an interesting theory that CK13 positive hillock cells could be increased massively in number when looked at in tumours.

Club and hillock cells as mentioned above may also have a role to play in the differentiation of prostate tissue. This is evidenced by PSCA positivity found both in hillock and club cells as well as luminal epithelial cells – half of whom show positivity for the marker as well. Studies by Tsujimura et al. and Goto et al. (2006) showed the enrichment of PSCA in proximal murine prostate but similar findings in humans could not be seen because of the significant anatomical variation between the two tissues.

1.12. DLK-1 positivity in prostate tissue

Following laser capture microdissection using PALM MicroBeam laser micro-dissection microscope (Leica Microsystems, UK), sequencing of the entire mitochondrial genome was done using a two-stage amplification workflow, as previously described by Taylor et al (2003). This was followed by RNA sequencing using the Illumina HiSeq2500 platform and then PCR amplification was done. Following characterisation of transcriptomes of cells thought of as stem cells by Moad et al., mRNA sequencing data showed DLK1 to be a highly upregulated transcript from these cells. Unfortunately, the study done by Henry et al. did not have DLK-1 as a marker that was looked for in their sequencing studies so it is hard to comment on any direct and obvious correlation. However, Ceder et al., in 2008 reported DLK1 to be a possible human stem cell marker

in situ. It codes for a cell surface protein which in turn serves as a dead ligand for the Notch pathway – a metabolic pathway that is important when it comes to homeostasis within the prostate epithelium. Immunofluorescence revealed colocalization of DLK1 with Alpha-6 integrin (CD-49f) which is an established basal cell marker and associated notch receptors.

The Notch pathway regulates basal cell differentiation from its progenitors. The pattern that was seen from immunofluorescence indicated that DLK1 provided an inhibitory signal when it came to basal progenitor differentiation in the niche. When it came to luminal cells, DLK1 provided an inhibitory signal to the Notch pathway yet again but with the different purpose of preventing Notch-regulated resistance to cell death through anoikis in the luminal compartment.

Focal expression of DLK1 was found in the basal epithelium situated between the urothelial boundary and the prostate epithelial boundary – typically going from the urethra to the proximal prostatic ducts. This extension happens in a zig zag pattern with prostate epithelial clonal apices around the circumference of the duct being backfilled by the urothelial encroachments, leading to an interdigitating appearance. (Moad et al., 2017)

To confirm the multipotent nature of said putative stem cells, further experiments were done with spheroid cultures (chosen particularly over the organoid cultures due to their property of not supporting luminal differentiation). Basal cell spheres that were DLK positive managed to be passaged beyond 3 generations with the sphere formation correlating with the size and number of the original founder generation. The DLK negative population did not survive serial passaging, diminishing in number before eventually exhausting itself post 6-8 weeks of culture – indicating a limited self-renewal capacity.

Post-6 weeks of culture, visible lumens were seen in the DLK positive cell based spheroids and these could be maintained beyond 8 weeks showing both acini-like and duct-like structures. They also continued to renew DLK positive cells and were able to regenerate the differentiated organisation of the prostate epithelium into basal and luminal cells. This histological differentiation occurred after 6 weeks of culture upto which even the DLK negative cell spheroids could generate amorphous spheroids showing that DLK1 was important during the process of differentiation.

1.13. Parallels to the Intestinal Stem Cell Niche

Stem cell niches, as mentioned before, have been described for other organs in the human body. The human intestinal stem cell niche is one of the most well described among them. Along with the zigzag pattern that was identified, the finding of long-ranging, cohesive clones from the stem cell niche in work done on the prostate by Moad et al. seems to relate in some respects to the intestinal stem cell niche as detailed below.

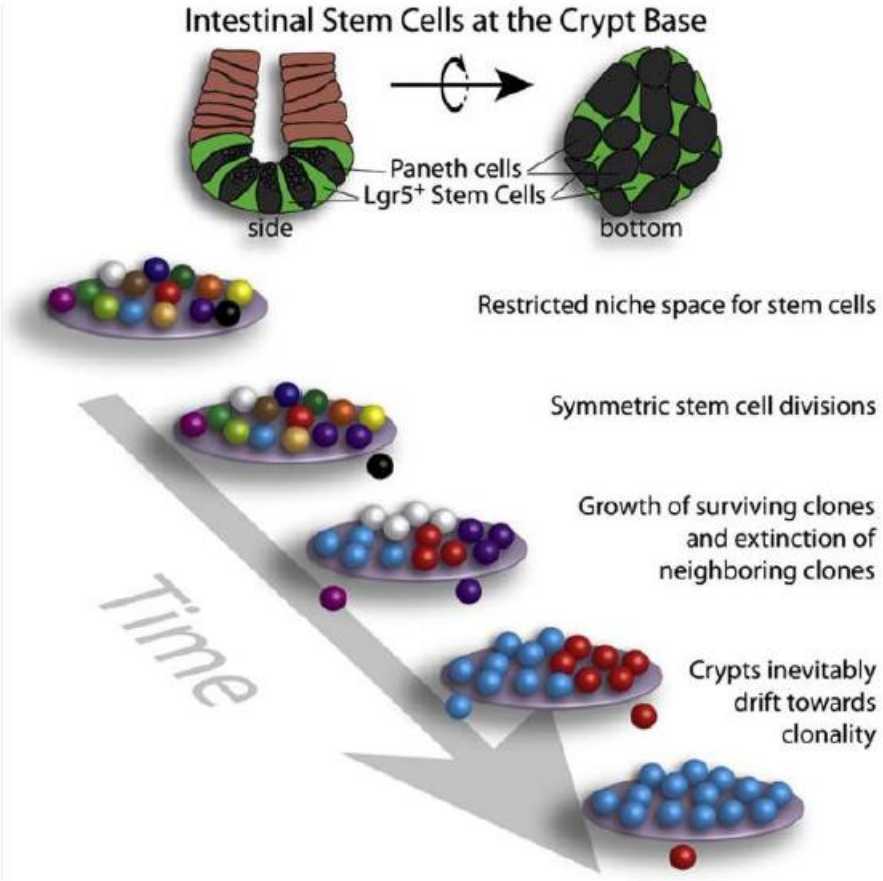


Figure 1.8 - Figure illustrating the clonal selection that occurs in intestinal stem cell niches allowing for the existence of a fixed number of stem cells within the niche.

In the small intestine, crypt base columnar (CBC) cells are the stem cells distributed throughout the crypt base and marked by positivity for Lgr5 expression. The niche is composed of the CBC cells plus the Paneth cells and the surrounding mesenchyme. The CBCs are about 14 to 16 in number and exist in neutral competition with one another to produce daughter cells. (Lopez-Garcia et al., 2010; Snippert et al., 2010; Ritsma et al., 2014)

There also exist another set of cells known as the quiescent or slow-cycling cells. These cells show stem-like characteristics but are normally quiescent in homeostasis, only being activated when injury requires replenishment of cells and can even be

recruited back into the stem cell population. They are at or near the +4 position from CBC cells.

Lineage tracing done on cells in the intestinal stem cell niche revealed that the CBCs gave rise to daughter cells – some of whom retained the stem cell qualities and were daughter CBCs and others of whom became transit-amplifying cells (TA cells). Some of the CBCs which were at the junction (known as border cells) ended up losing their stem capabilities – characterised by the loss of expression of Lgr5. This helped maintain a constant number of CBC population even through cell division and the production of daughter cells. This mechanism ensures a constant displacement of cells out of the niche – and helps keep the number of the stem cell intact within the stem cell compartment of the ISC niche. In these studies it became clear that stem cells closer to the niche boundary tend more likely to be replaced while those that are further away from the boundary tend to experience survival within the niche as a stem cell.

This can be linked in certain aspects to the human prostate but before we do that, some things have to be kept in mind. The intestinal stem cell niche is well characterised which cannot be said of the prostate stem cell niche. The stem cells in the prostate are DLK1 positive and their location is said to be proximal – at the junction between the urothelium and the prostate epithelium. The small intestine is an organ that experiences a very high turnover of cells as well when compared to the prostate. This means that potentially both the number of stem cells and their activity will be more in number when compared to the prostate.

However, what can again potentially be extrapolated from data obtained by Moad et al, previous studies done on murine prostate and the intestinal stem cell niche mechanics is that stem cells from the basal region in the proximal ducts of the prostate give rise to cells further along and as they migrate distally they lose their stemness in a manner similar to CBCs. The presence of the occasional unipotent luminal stem cell as seen by Moad et al. could also indicate that maybe some of these cells also retain a certain amount of stemness in them and could give rise to luminal cells in case of injury. What this also could potentially account for is the peripheral and focal presentation of cancer within the prostate – as expanded upon next.

1.14. Stemness as a concept in relation to cancer

Batlle and Clevers in 2017 recognised that in cancers, a distinct type of cells form a part of the disease influencing the progression of the disease itself. These cells were

called cancer stem cells. Defining what constitutes a cancer stem cell is something that inherently has difficulties as these cells behave differently in different types of cancers and there is no situation whereby one definition can suitably cover all of them.

According to Laplane and Solary (2019), stemness can be an umbrella term used to talk about four different kinds of properties for cells that fall under the bracket of possessing stem capabilities.

1. A Categorical Property: An intrinsic feature independent of interaction with the surrounding microenvironment – a property now applicable only to certain cancers.
2. A Dispositional Property: Stemness that shows itself only when interacting with the outside or surrounding environment. This outside or surrounding environment is the niche microenvironment – which becomes important in distinguishing it from stemness being a systemic property.
3. A Relational Property: Relies on the nature of interaction between two separate entities. There is no set rules for stemness if it is a relational property as opposed to a dispositional property where the outside environment determines its course of action in a particular direction.
4. A Systemic Property: A situation where the external environment determines the acquisition of stemness by non-stem cells. Bypassing the requirement of a particularly engineered niche is one of the characteristics of this.

Cancer stem cells can be understood to be the basis of the disease in any organ as the ability to reproduce without control is one of the characteristics of cancer. Cancers usually also involve undifferentiated cells more than differentiated ones, pointing somewhat to a regression towards stem or stem-like capabilities.

Prostate cancer presents as a focal disease in the peripheral regions of the prostate. The role of the stem cell in prostate cancer is as yet undefined. Based on the above reclassification of stem cell behaviour, one can speculate on how stem cells present in the basal region of the proximal part of the prostate as well as sparse luminal unipotent stem cells can affect cancer.

One possibility involves Knudson’s two-hit hypothesis whereby the first hit (or the first mutation) takes place in the proximal duct itself but is not enough to turn the cell cancerous. As the stem cell migrates distally – turning into a basal or luminal

phenotype – it acquires the second mutation (second hit) – thereby turning it cancerous.

Another possibility is that while both mutations could be acquired early on while the cell is in the proximal region, the conditions outside the cell (within the niche environment) are not conducive to cancer formation. But once they have migrated distally to the peripheral regions of the prostate, the conditions there may be suitable for cancer development and the stem cell that has acquired the mutations early on may finally be able to produce focal tumours.

The third potential mechanism may be that the unipotent luminal stem cells identified by Moad et al. may end up acquiring mutations that turn them cancerous. As these are already peripheral, they would present as prostate cancer in peripheral locations.

The first and third instances would point towards cancer formation being a categorical property where it depends solely on the mutations being acquired rather than anything else in the external environment while the second instance would point to the formation of cancer itself being a systemic property. While it is unclear which of these is the right answer or whether all of these are mechanisms which account for the focal and peripheral presentation of most prostate cancers, it is an interesting avenue to pursue and the understanding will come when the stem cell niche is characterised both structurally and functionally.

This project will investigate the structural aspect of the stem cell niche. Specifically, using immunohistochemical and immunofluorescence techniques, the study will be looking at identifying the location of the stem cells – using known stem cell markers such as DLK-1 – and map out the relationship between prostate epithelium and urothelium. Following this, the plan is to perform 3D reconstruction using specific software for the purpose and generate a three-dimensional image of the stem cell niche and the surrounding microenvironment.

1.15. Hypothesis

The hypothesis is that there exists a stem cell niche within the confines of the human prostate – located at the juxtaposition of the urothelium and the prostate epithelium whose structure extends deeper into the tissue and can be marked out using stem cell-specific markers such as DLK-1 in order to form a complete understanding of the stem cell niche microenvironment.

The stem cell niche involves interaction between the prostate epithelium, the urothelium and the stem cells themselves. Hence, having a combination of cellular markers for each of these individual tissue types on a single slide would show the location of stem cells and a three-dimensional reconstruction of a sequential number of slides should then demonstrate the stem cell niche from a structural point of view.

1.16. Objectives

1. To optimise the list of markers to be used to identify the prostate epithelium, the urothelium and the stem cells
2. The use the Opal kit to multiplex a combination of these markers and generate images that could help us identify the stem cell niche

Chapter 2: Materials and Methods

2.1. Tissue Samples

These prostate samples were mainly from men with non-muscle invasive bladder cancer. Most have had primary cystectomy, but in cases that haven't, there may be expected to be an effect from intra-vesical mitomycin C or BCG (clinically we know these treatments are ineffective in Transient Cell Carcinoma involving the prostate tissue). In the cases of muscle invasive bladder cancer, many had primary cystectomy. But a few did have neoadjuvant chemotherapy – this is cisplatin based which is ineffective in the treatment of prostate cancers and we speculate not to significantly change the epithelial homeostasis – however I will bear all these treatments in mind when undertaking the analyses. The formalin protocol was standardised, in line with routine pathology approaches in Newcastle Hospital labs. The Prostates were soaked in formalin overnight. Other approaches are available, such a freeze fixing but architecture is not preserved as well and limits its use in this study.

The ethical consent and informed consent of the patients were taken in line with approved regulatory processes and the tissue was collected under the Newcastle University Tissue Act licence under the name of Rakesh Heer and a specific urology project

2.2 Immunohistochemistry

Immunohistochemistry was performed on 5µm sections of formalin fixed paraffin embedded (FFPE) tissue. The sections were deparaffinised in xylene and followed by hydration with six serially decreasing concentrations of ethanol (from 100% to 50%) before ending with running water. Sections underwent heat-induced epitope retrieval by heating using a decloaking chamber. The buffer used was the 0.01M citrate buffer (pH 6.0). The components of the citrate buffer are shown below in Table 2 The slides were exposed to temperatures of 121°C for a period of 30 seconds. Immersion of slides into 3% hydrogen peroxide for 10 minutes was used to remove endogenous peroxidase activity.

Table 2. Required components for citrate buffer

Component	Amount	Concentration
Sodium Citrate dihydrate (mw: 294.10 g/mol)	24.269 g	0.0825 M
Citric Acid (mw: 192.12 g/mol)	3.358 g	0.0175 M

pH adjusted using 0.1 N HCl	As required	
Distilled water	To make 1 L solution	

After the addition of blocking buffer (2.5% horse serum) for 20 minutes, the following primary antibodies were added to separate slides and incubated for 1 hour at room temperature:

Table 3 – List of antibodies used for IHC optimisation experiments

Antibody	Type	Source	Product Reference Number
Anti-UPK1b	Rabbit polyclonal	Sigma Aldrich (Leica Biosystems)	HPA031799
Anti-p63	Rabbit Polyclonal	BioLegend	619002
Anti-34βe12	Mouse Monoclonal	Aglient Dako	M0630
Anti-CK8+18	Mouse Monoclonal	Abcam	Ab17139
Anti-PSA	Rabbit Monoclonal	Aglient Dako	A0562
Anti-AR	Mouse Monoclonal	BD pharmigen	554225
Anti-DLK1	Mouse Monoclonal	Abcam	Ab89908
Anti-Ki67	Mouse Monoclonal	Novocastra (Leica Biosystems)	NCL-L-Ki67-MM1

The primary antibody was substituted with 4% Bovine Serum Albumin (BSA) in Phosphate Buffered Saline (PBS) for negative controls. The slides were washed in Tris-Buffered Saline (TBS)/Tween and the secondary antibody added (polymer Horse Radish Peroxidase-labelled antimouse/rabbit/goat, ImmPRESS™) for 30 minutes. Slides were rinsed with running water and placed again into TBS/T. Diaminobenzidine (DAB) peroxidase substrate solution (Vector) was applied to slides for 5 minutes and removed by rinsing in running water to expose the bound peroxidase. Slides were rinsed in running water following which Gill's haematoxylin was used to stain the nuclei and Scott's tap water was used as a blueing reagent.

The stained sections then underwent dehydration with six serial immersions in increasing concentrations of ethanol (from 50% ethanol to 100% ethanol), followed by three immersions in xylene. Slides were mounted using distyrene plasticiser xylene (DPX) and a coverslip placed over the tissue section.

2.3. Immunofluorescence

The immunofluorescence protocol followed the same steps as the IHC protocol till the blocking step (i.e. the dehydration steps, the use of the decloaking chamber, exposure of slides to high temperatures, use of the citrate buffer and the use of hydrogen peroxide to nullify endogenous peroxide activity). At this point the blocking buffer (4% BSA in PBS) was added to the slides for 1 hour. Following this, the primary antibodies were added and kept for overnight incubation at 4°C. The primary antibodies used were the same as for immunohistochemistry. 4% BSA in PBS in. Slides were washed in PBS then incubated in the dark for 30 minutes at room temperature with the corresponding secondary antibody (Goat-anti-mouse IgG conjugated to Alexa Fluor 488 or Donkey-anti-rabbit IgG 21 conjugated to Alexa Fluor 568, 1:400 dilution – Thermo Fisher Scientific). Slides were washed in PBS then stained with DAPI (Vector Laboratories, Burlingame, CA). Slides were visualised and images acquired using fluorescence microscopy (Leica DM6).

2.4. Opal Kit

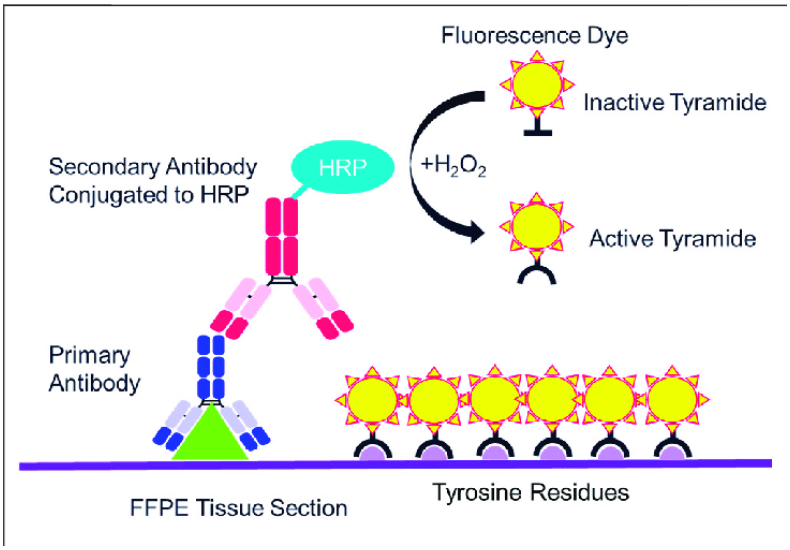


Figure 2.1 - Mechanism of tyramide signal amplification (TSA) staining. Opal dyes allow for the use of any standard unlabeled primary antibody, including multiple antibodies raised in the same species. After introduction of the primary antibody, the Opal polymer HRP is applied. The Opal system uses TSA to amplify IHC detection by covalently depositing multiple fluorophores near that targeted antigen. After labelling is complete, antibodies are removed in a manner that does not disrupt the Opal fluorescence signal, allowing for the next target to be detected without antibody cross-reactivity. Image adapted from C. Hoyt, 2021.

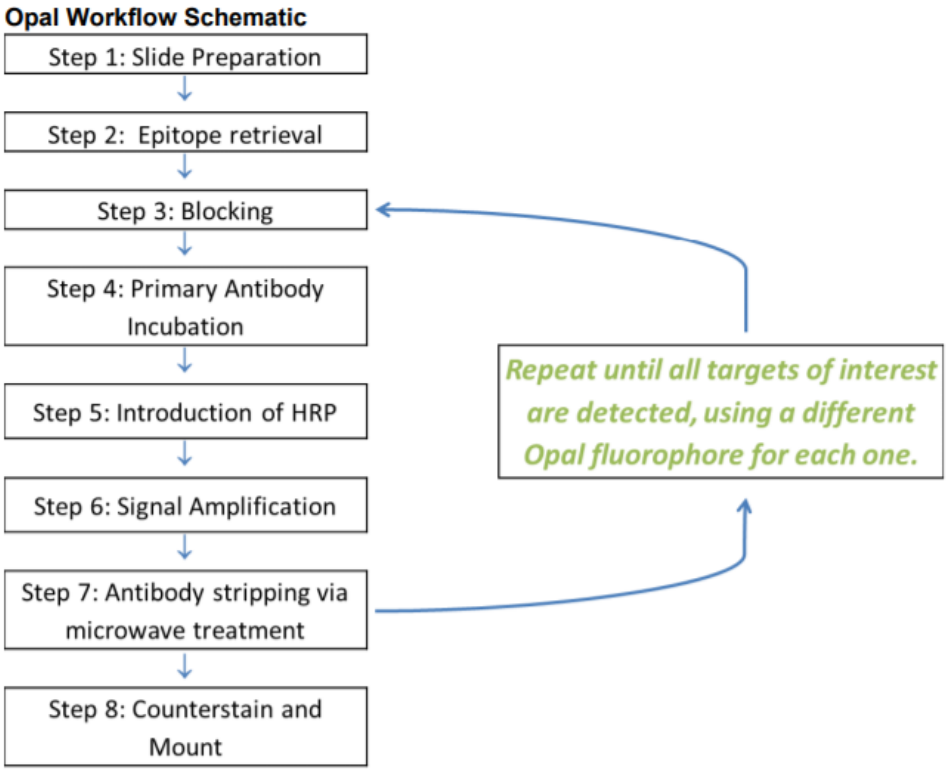


Figure 2.2 – The experimental workflow using the Opal kit – a flowchart.

The Opal kit comes in a pack- with dry Opal reagents meant to be stored at -20°C. If reconstituted with Dimethyl sulfoxide (DMSO), it must be stored at 2 to 8°C. The Opal method allows for detection of multiple biomarkers in a single section of tissue – more on this is discussed in the Discussion section. A few solutions need to be prepared before the procedure can go ahead.

AR6 BUFFER WORKING SOLUTION: Dilute 10X AR6 buffer at 1:10 with peroxidase free water.

PRIMARY ANTIBODY WORKING SOLUTION: Dilute the primary antibody in PerkinElmer Antibody Diluent/Block at optimal concentration (determined by IHC with DAB)

SECONDARY ANTIBODY WORKING SOLUTION: Opal Polymer Horseradish Peroxidase (HRP) Mouse + Rabbit is supplied as a ready to use solution

OPAL FLUOROPHORE WORKING SOLUTION: Reconstitute each Opal fluorophore in 75µl of DMSO. Before each procedure, dilute Opal fluorophore in 1X Amplification Diluent to make Opal Fluorophore Working Solution. It is recommended to start diluting the Opal fluorophore at 1:100. The assay should be optimised according to the Opal

Assay Development Guide. Generally, 100-300µl of Opal Working Solution is required per slide. Any unused portion of this solution is to be discarded.

Preparation of tissues or cells for detection with the Opal kit takes place using standard fixation and embedding techniques. Each slide must be baked in the oven for at least 1 hour at 65°C. The slides are then dewaxed with Xylene (3X10 mins) followed by rehydration through a graded series of ethanol solutions from 100% ethanol to 50% ethanol. After rehydration, the slides are rinsed briefly with distilled water. The slides are then placed in a jar which can be used to heat in the microwave with the appropriate slide holder, fill it completely with the AR buffer and loosely cover the jar with a lid. It is then placed in the microwave for 45 seconds at 100% power and then microwave for an additional 15 minutes at 20% power. The slides are allowed to cool down to room temperature (15-30 minutes) rinsed in distilled water followed by TBST. Using a hydrophobic pen a barrier is made around the tissue section on the slide. The tissue sections are then covered with blocking buffer and incubated in a humidified tissue chamber for 10 minutes at room temperature. After draining off the blocking buffer and applying primary antibody working solution, the slides are incubated according to optimised conditions within the lab – either overnight at 4°C or at room temperature for an hour. The sections must be completely covered. The slides are rinsed in TBST by washing the slides in 3X2 mins TBST at room temperature preferably with agitation. The slides are incubated in Polymer HRP Ms+Rb for 10 minutes at room temperature. The slides are rinsed again in TBST and washed in 3X2 mins TBST at room temperature preferably with agitation. Excess wash buffer is drained off. 100-300µl of Opal Fluorophore Working Solution is pipetted onto each slide. Slides are then incubated at room temperature for 10 minutes. The slides are rinsed and washed again in TBST like in previous steps. The above steps are repeated for all markers to be identified. Once all biomarkers have been identified, DAPI Working Solution for 5 minutes at room temperature in a humidity chamber must be applied. The slides are then washed for 2 minutes in TBST buffer and then 2 minutes in water. Coverslip the slides with mounting medium

Imaging of the slides was done with the fluorescence microscopy (Leica DM6) at appropriate wavelengths.

2.5. SIMPLE: Sequential Immunoperoxidase Labelling and Erasing

The Opal kit had various problems associated with multiplexing different markers including photobleaching, epitope denaturation and more which are discussed further below. Upon research, a method published by Glass et al. (2009) caught my interest as while SIMPLE was a method to visualise multiple markers within the same section of tissue, it uses an alcohol-soluble immunoperoxidase substrate 3-amino-9ethylcarbazole (AEC) and imaging and elution at every stage of the process. The procedure is as follows.

Paraffin sections mounted on slides were placed in a 60°C oven for 1 hour. The slides were then dewaxed through xylenes (similar to IHC protocol) and rehydrated through a graded series of alcohols from 100% ethanol to 50% ethanol followed by distilled water. Prior to immunostaining the slides were stained with Gill's haemotoxylin for 15 seconds and then counterstained with Scott's tap water for 30 seconds.

Slides were then coverslipped in aqueous mounting media (70% glycerol in PBS). The slide was then imaged with the Aperio imaging system. Decoverslip the slides by immersion in distilled water followed by TBST. Using a commercial microwave, antigen retrieval was performed in a 10mM sodium citrate buffer (pH 6.0). Allow the slides to cool to room temperature. Endogenous peroxide activity is then quenched with 3% hydrogen peroxide for 10 minutes followed by rinsing in TBST. Block the tissue in 2.5% normal horse blocking serum (Vector Laboratories). Incubate the slides with the primary antibody for 1 hour at room temperature or overnight at 4°C. Detection of the primary antibody was performed with appropriate ImmPress reagents for mouse or rabbit primaries. AEC was used to give the colour to the primary antibody detected

Slides were then coverslipped in aqueous mounting medium and imaged using the Aperio Scanscope system. After imaging, slides were decoverslipped in distilled water and TBST and dehydrated through a series of ethanols up to 95% concentration. Slides were incubated until no visible AEC reaction product remained. Following rehydration, antibodies were eluted by incubating sections in 0.15 M $KMnO_4$/0.01 M H_2SO_4 solution for 2 minutes. Immediately this was followed by a by a distilled water wash. Tissue was then restained, beginning with the blocking step, as described above.

2.6. DAB-SIMPLE

This method was developed as a corollary to SIMPLE where the testing of multiple markers can be done with the use of DAB instead of AEC and it eliminates the need for elution. More is discussed later.

The key difference between this method and SIMPLE in terms of procedure is the use of DAB (used in IHC described in section 3.1) instead of AEC. The procedure is otherwise identical even for multiple markers.

2.7. IHC for multiple markers

Similar to the idea of SA-SIMPLE, IHC could be done for multiple markers with the same protocols as in 3.1 and using the same methods for multiple marker identification as in SA-SIMPLE (Section 3.5). The only difference is that instead of AEC, the marker that was used in this case was AMEC-Red. The reasoning behind the selection of AMEC-Red is discussed later. The tissue was also subjected to heat mediated antigen retrieval using a decloaker every time a new marker needed to be stained.

Chapter 3: Results

The objective of this study is to map out the structural architecture of the prostate stem cell niche and its components. To do that, each of the tissue types that make up the niche need to be marked out and visualised on individual FFPE slides before performing 3-D reconstruction to visualise the niche in its physiological state.

The choice of methodology therefore is to use immunohistochemistry to optimise the appropriate markers followed by the use of the Opal kit to demarcate and visualise multiple markers on the same FFPE slide of benign prostate tissue.

3.1 Optimisation for IHC

A list of candidate markers was taken which would mark out prostate and urothelial tissue. For prostate tissue a wide variety of markers were taken which marked out luminal or basal cells and were either nuclear or cytoplasmic while for urothelial tissue it was decided to go ahead with uroplakin 1b which marked out both the superficial and some of the deeper layers of the urothelium as opposed to the other uroplakins which marked out only the superficial layers. The full list of candidate markers is shown in Table 2 above.

The results of all of these optimisation experiments shown below demonstrate the various localisations of these markers within prostate or urothelial cells as the case may be. The optimisation process involved looking at different dilutions of a particular marker and then choosing the best dilution in terms of localisation as well as staining pattern (i.e. the absence of overstaining or understaining). An example is shown below with PSA.

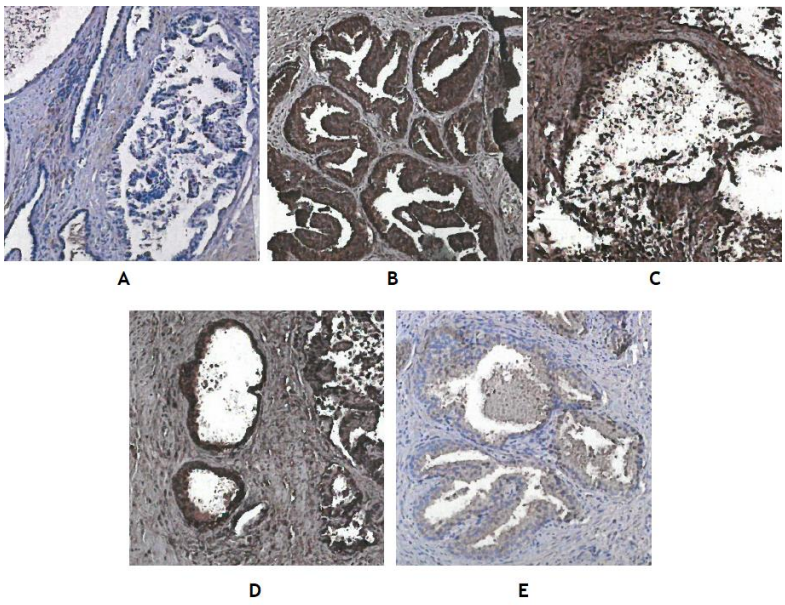
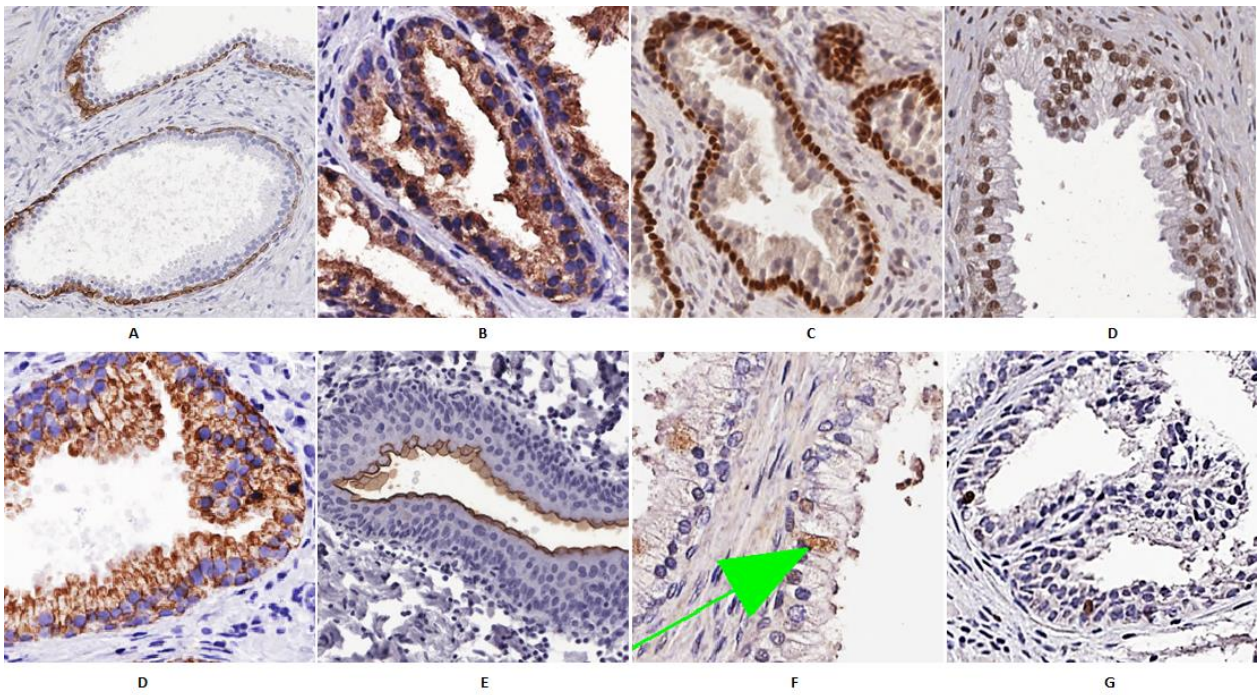


Figure 3.1. A to E – Testing various dilutions of PSA to determine the ideal concentration. A – Negative control B. 1:100 C. 1:500 D. 1:1000 E. 1:5000. 1:5000 was determined to be ideal.

Because two different tissues (prostate and urothelium) were involved, it made sense to test these markers on both of these tissue samples and hence uroplakin 1b for example was also tested on the prostate and PSA and other prostate markers were tested on the urothelium.

Of the findings it is important to note that 34BetaE12 marked out only the basal layer in the prostate but marked out both superficial and deeper layers in the urothelium while CK8+18 marked out only the luminal layer in the prostate and marked out all the layers in the urothelium as well. Ki-67, AR and PSA are shown in the same section as examples of markers that do not stain positive in the urothelial tissue while uroplakin also demonstrates non-staining in the prostate tissue making them exclusive to the tissues they stain positively in.

NKX3.1 is not shown among the results section because the antibody used did not generate results satisfactory enough to clearly say that it marks out NKX3.1. It was also deemed redundant as a marker for prostate luminal nuclei as AR is a better marker and has shown up clearly in immunohistochemistry monoplexes. However, due to the differences in the protocols of the Opal kit and IHC, it was decided to carry forward NKX3.1 to the Opal monoplex stage just to see if that made any difference to how well marked out it was.



Figures 3.2. A to H – Images from IHC optimisation of various antibodies against different cellular markers in the prostate (and urothelium for UPK-1b) with their optimal concentrations. A. 34BetaE12 – (Optimised at 1:100 concentration) B. PSA (Optimised at 1:5000 concentration) C. p63 (Optimised at 1:100 concentration) D. Androgen Receptor (Optimised at 1:100 concentration) E. CK8+18 (Optimised at 1:100 concentration) F. UPK-1b (Optimised at 1:20 concentration) G. DLK-1 (Optimised at 1:100 concentration) H. Ki-67 (Optimised at 1:100 concentration)

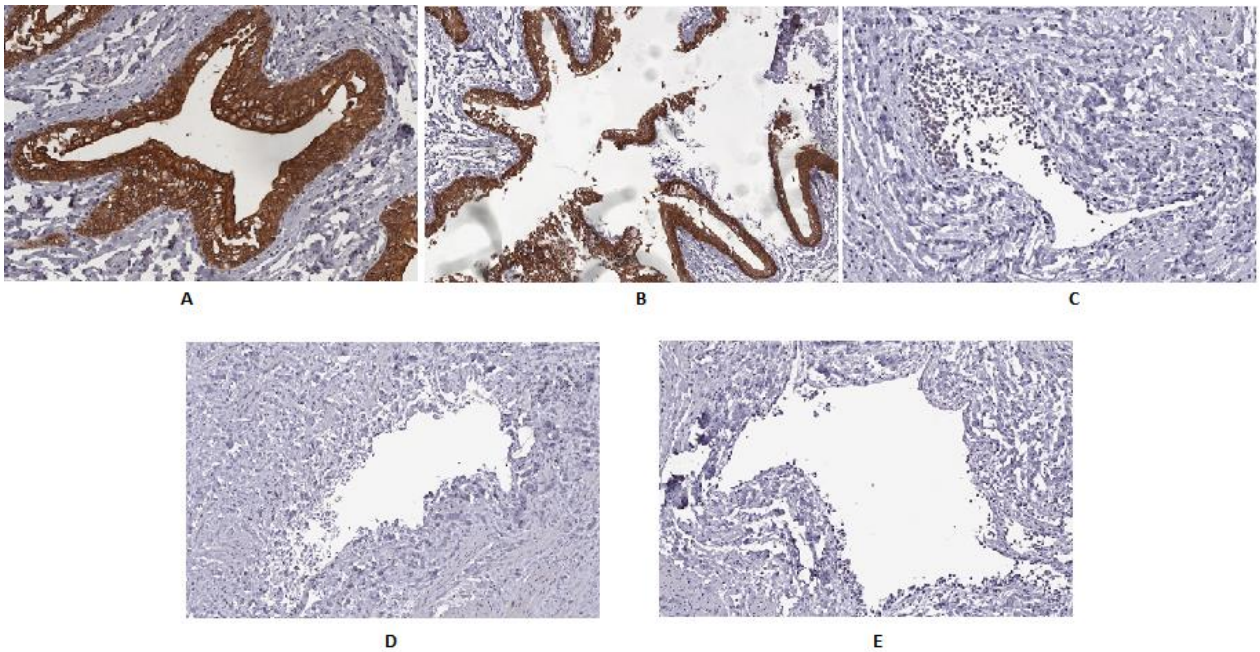


*Figure 3.3 A to E – Testing of prostate markers at their optimal concentrations on ureteric tissue. A. Ck8+18 (1:100) B. 34BetaE12 (1:100) C. Ki-67 (1:100) D.*AR (1:100) E. PSA (1:5000)

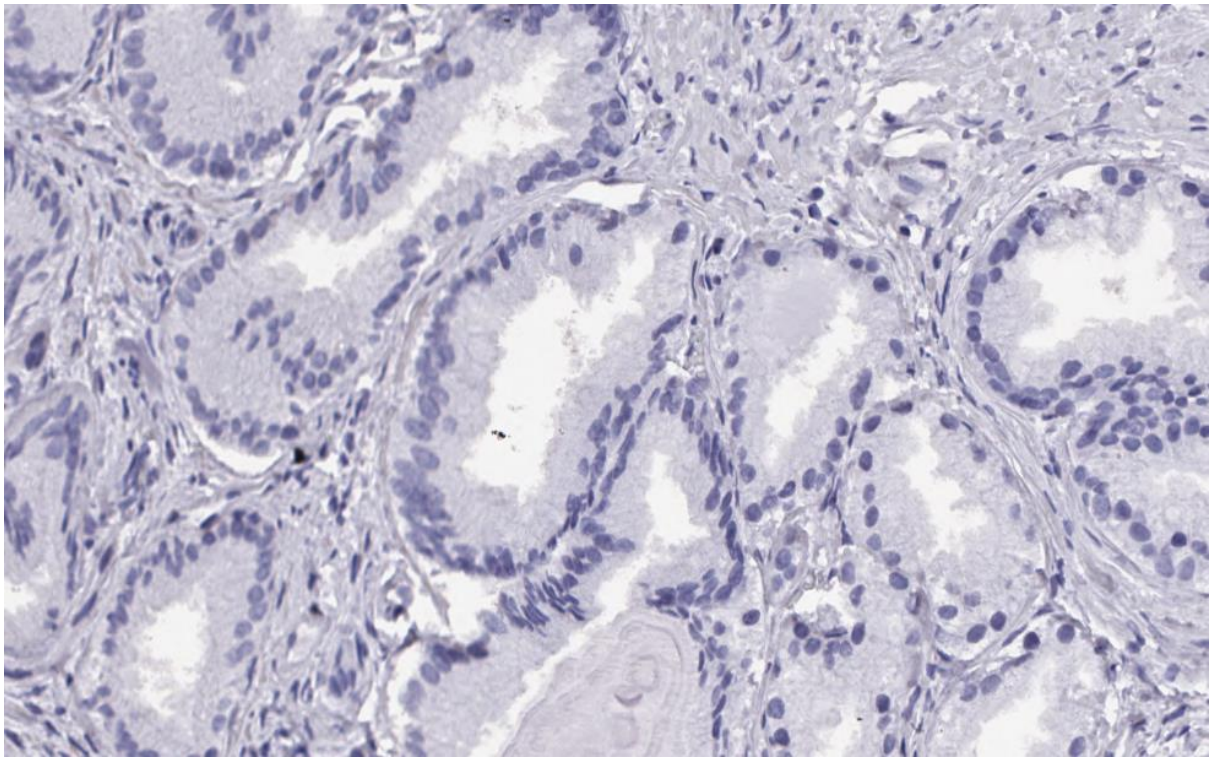


Figure 3.4 – Testing of urothelial marker UPK-1b on prostatic tissue.

3.2. Optimising Immunofluorescence

Immunofluorescence experiments were done not with the aim of separating markers further but with the aim of learning the technique of immunofluorescence and operating different light filters on the Leica DM6 fluorescent microscope. The technique only enabled me to learn the ropes when it came to operating microscopes. Hence not too many markers were done with IF and PSA and CK8+18 are shown in the results sections. Also the uroplakin Ib was also tried on the prostate section to show an absence even on IF.

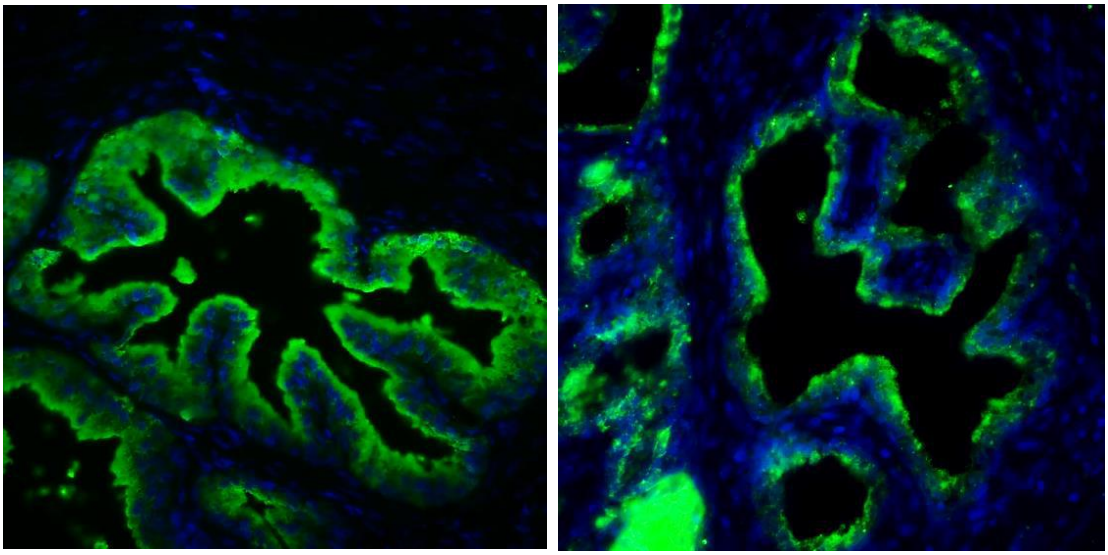


Figure 3.5 - PSA (L) and CK8+18 in IF at the same concentrations as used in IHC

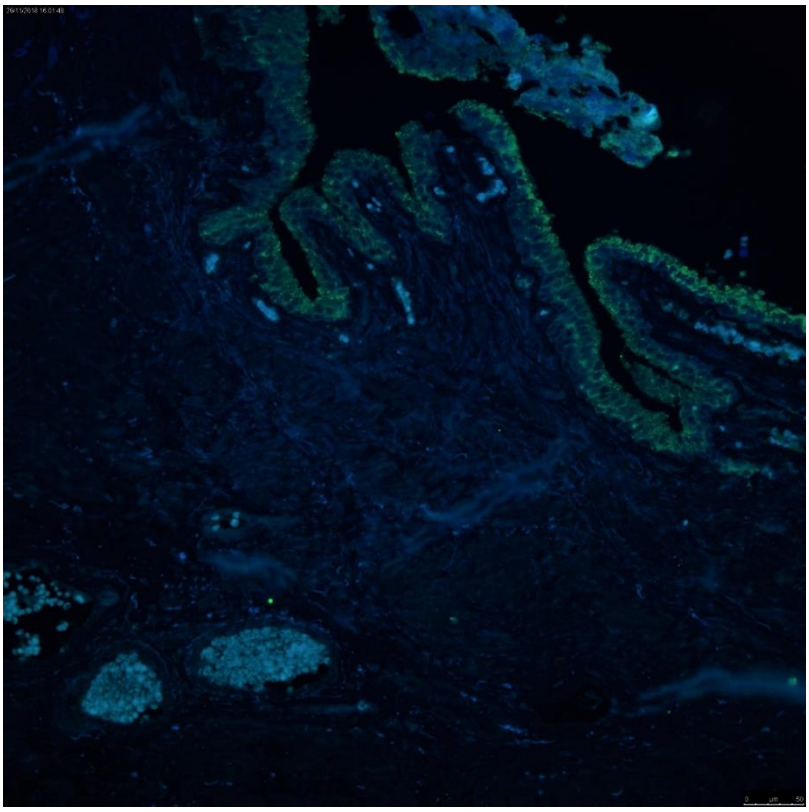


Figure 3.6 - PSA shown coloured green on a prostate section (1:5000 concentration)

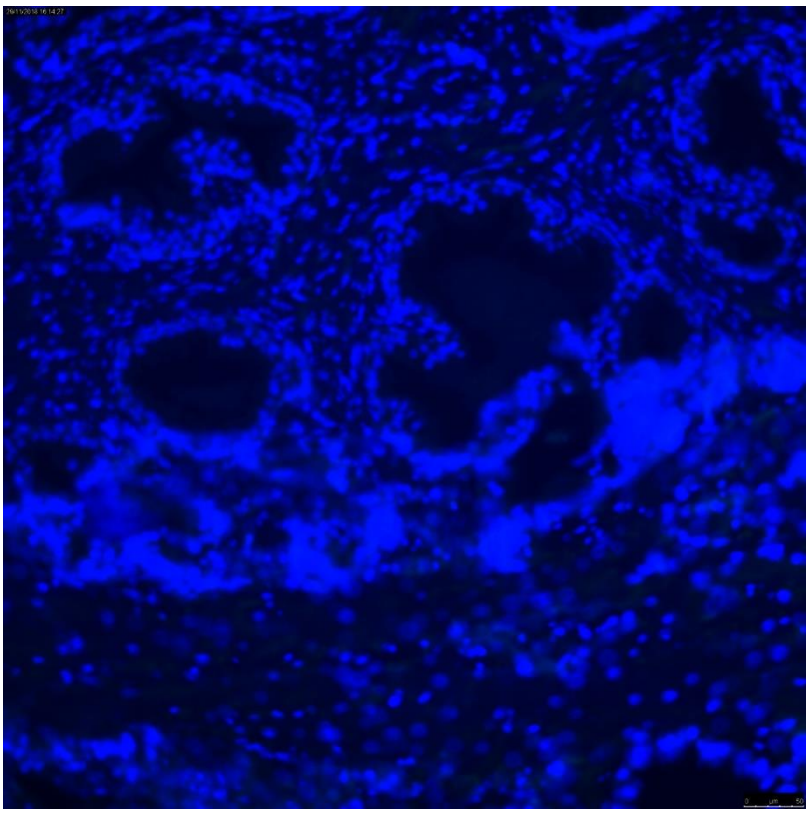


Figure 3.7 - UPK on Prostate section (1:20 concentration)

3.3. Opal Monoplex Optimisation

The commercial Opal kit is manufactured by PerkinElmer, USA and is available for performing manual multiplex IHC on up to 50 slides with the capability of identifying 4 or 7 markers. In this study the kit that was used is the Opal™ 7-Color Manual IHC Kit 50 slides.

The markers optimised with IHC were carried on with the same concentration to the Opal kit where they were validated again individually. While doing the monoplex for the Opal kit with each of the markers in the table above, it was established that the same concentrations that worked for DAB-based IHC would also work optimally for the Opal kit. NKX3.1 also responded better to the Opal kit and marked out luminal nuclear cells clearly. The other markers also worked well. P63 on the other hand was a marker that did not respond well at all to the Opal protocol. It was non-specific. The only concern with DLK-1 which seemed a little more specific with the DAB-IHC protocol than with the Opal protocol. However, it was still carried forward to the Opal multiplex experiments.

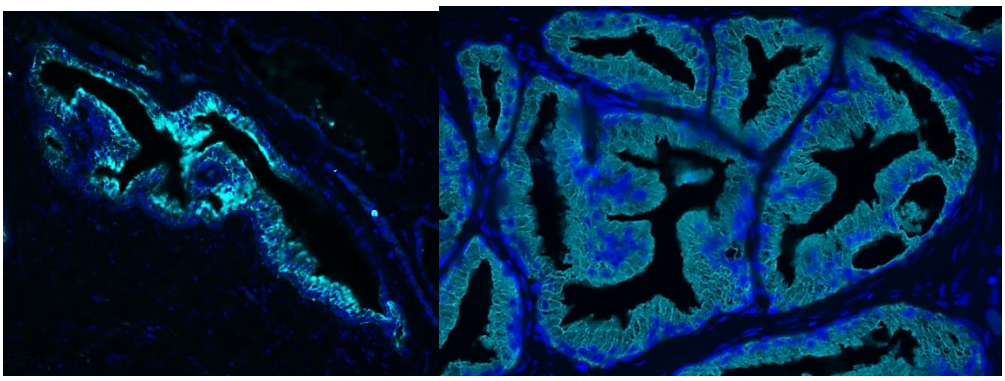


Figure 3.8 - CK8+18 and PSA Opal monoplex (Concentrations at 1:100 and 1:5000 respectively)

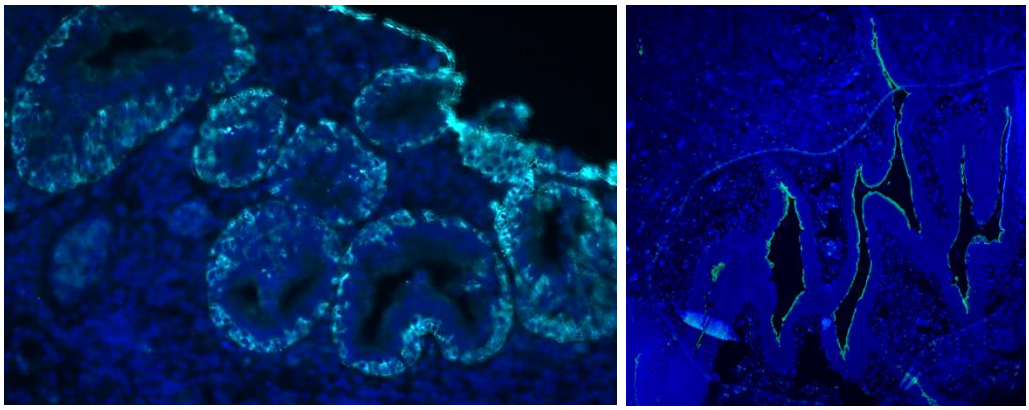


Figure 3.9 - 34BetaE12 on prostate tissue sections and UPK 1b on urothelium (1:100 and 1:20 respectively)

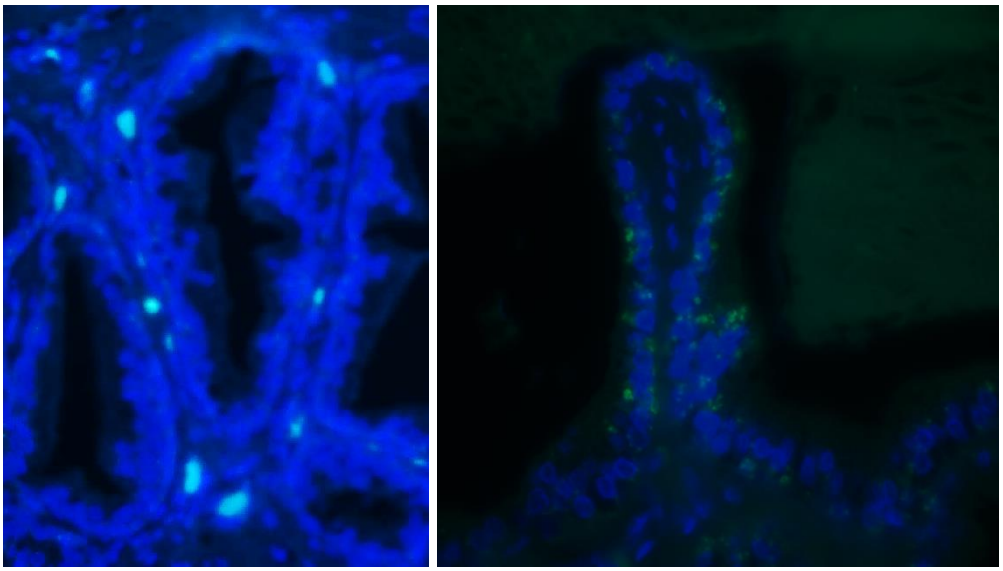


Figure 3.10 - Ki-67 and DLK-1 (both 1:100 concentration). DLK-1 was not effective in marking out only DLK-1 positive cells of the prostate

3.4. Opal Multiplex Optimisation

Many multiplex experiments were carried out with different combinations of markers (examples are seen in Table 4 and 5 below). Carrying out multiplex experiments

allowed us to determine the right combination of markers in order to define the stem cell niche. At the very outset, it was determined that a prostate basal marker, a prostate luminal marker, a stem cell marker and a marker for urothelial tissue were prerequisites.

However, following problems doing multiplex imaging such as the one demonstrated below and discussed further in the Discussion regarding the Opal multiplex experiments, the list of markers was eventually shortened to just PSA and UPK with the aim of demonstrating boundaries and interactions between the urothelium and prostate epithelium. The reasoning for this is discussed further below.

Furthermore, some of the slides were also in serial order and gave an idea of what the interaction between the urothelium and prostate epithelium looked like as we explored deeper into the tissue.

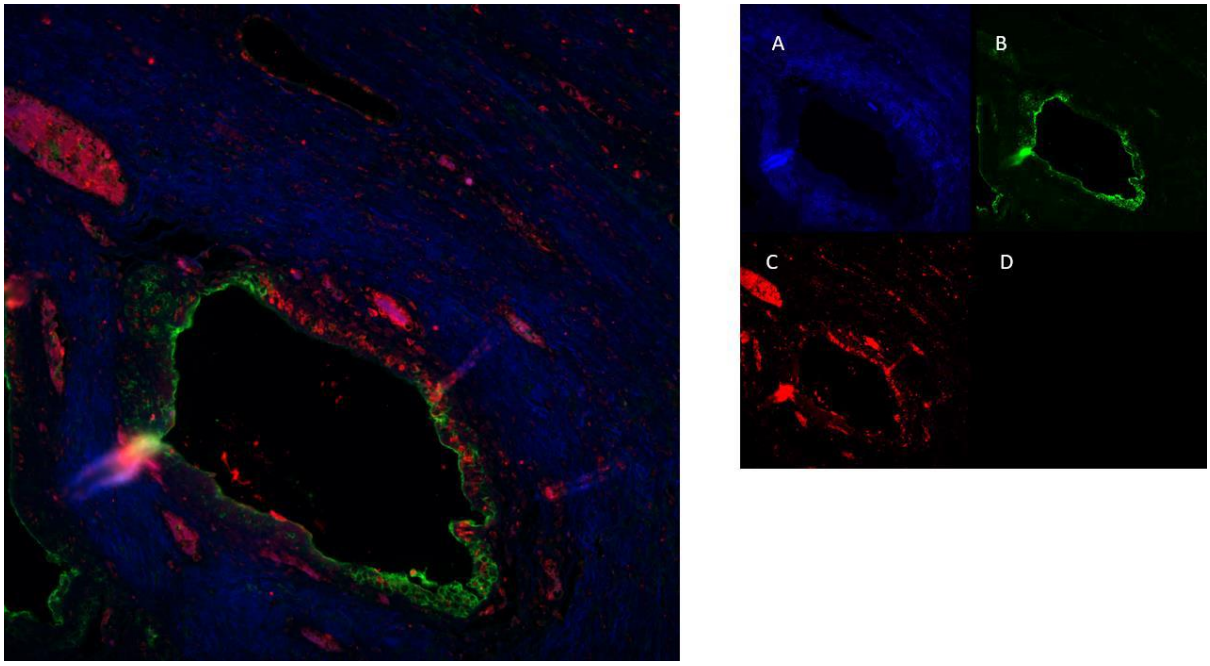


Figure 3.11 - The result from one of the early multiplex experiments done. The image on the left shows the composite image made up of individual markers. Shown on the right are staining pictures of these markers individually. A – DAPI B- PSA C- UPK D- 34BetaE12. 34BetaE12 in this image has completely not shown up and this highlights the problem of heat stability further discussed below.

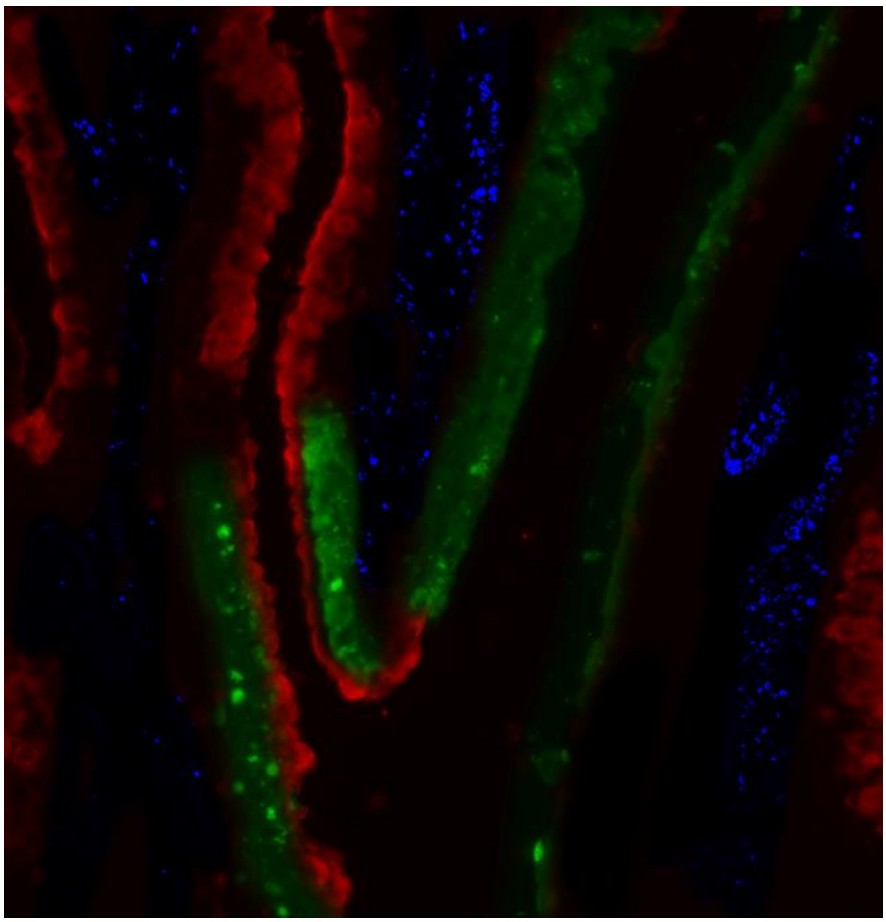
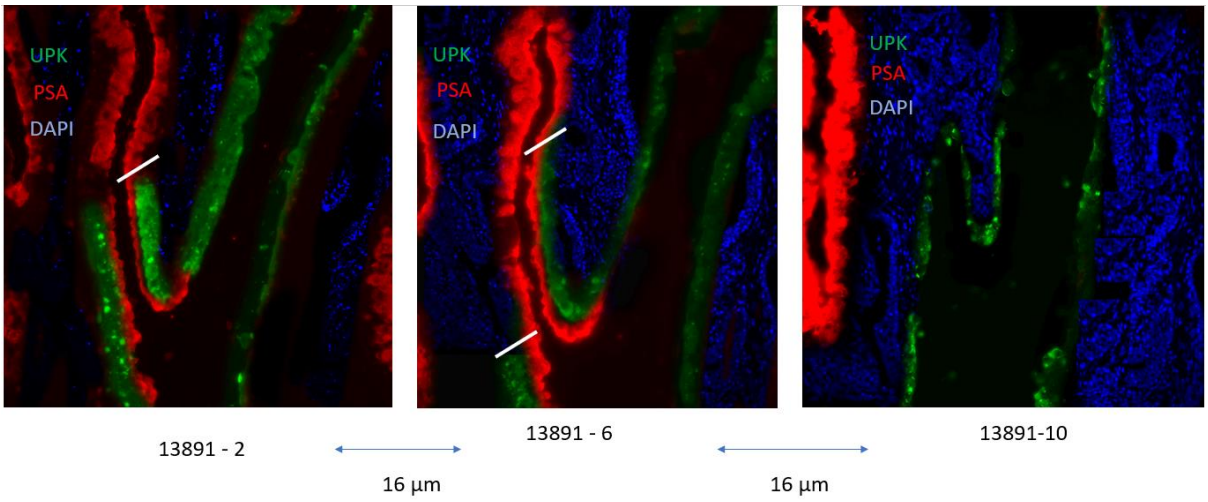


Figure 3.12 - An example of a multiplex Image of Uroplakin (green) and PSA (red) + DAPI (blue)



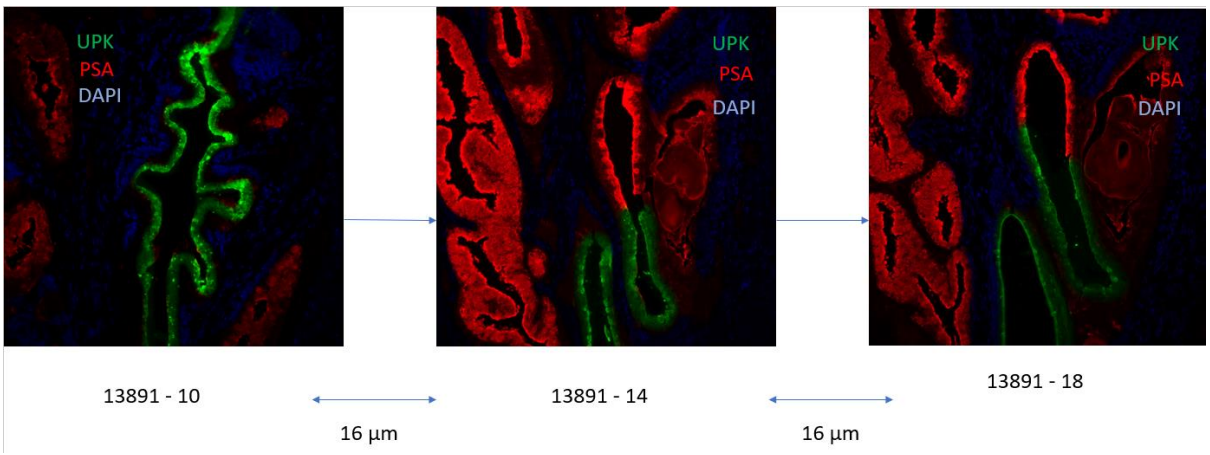


Figure 3.13 - Figures above show multiplex imaging with PSA (red), uroplakin 1b (green) and DAPI (blue) across three consecutive slides from the same patient but in two different regions of the sections. Each of these sections goes deeper into the tissue but still remains within the general vicinity of the prostate epithelium-urothelium junction. In both these sections, it is evident to see that there is some degree of overlap between PSA and UPK-1b which then separate from each other to become prostate and urothelial tissue independent of each other. The white lines represent a border between the two different tissue types.

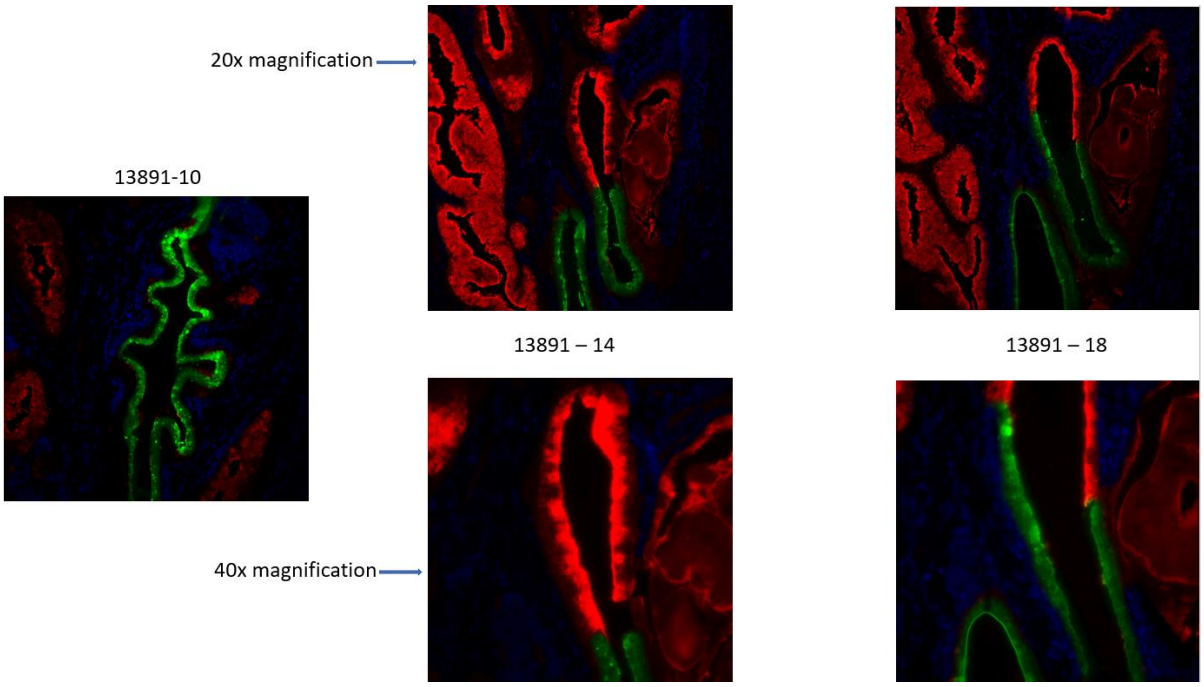


Figure 3.14 - The images above show the evolution of a prostate-urothelial overlap into prostate and urothelial tissue independent of each other in both low and high magnification.

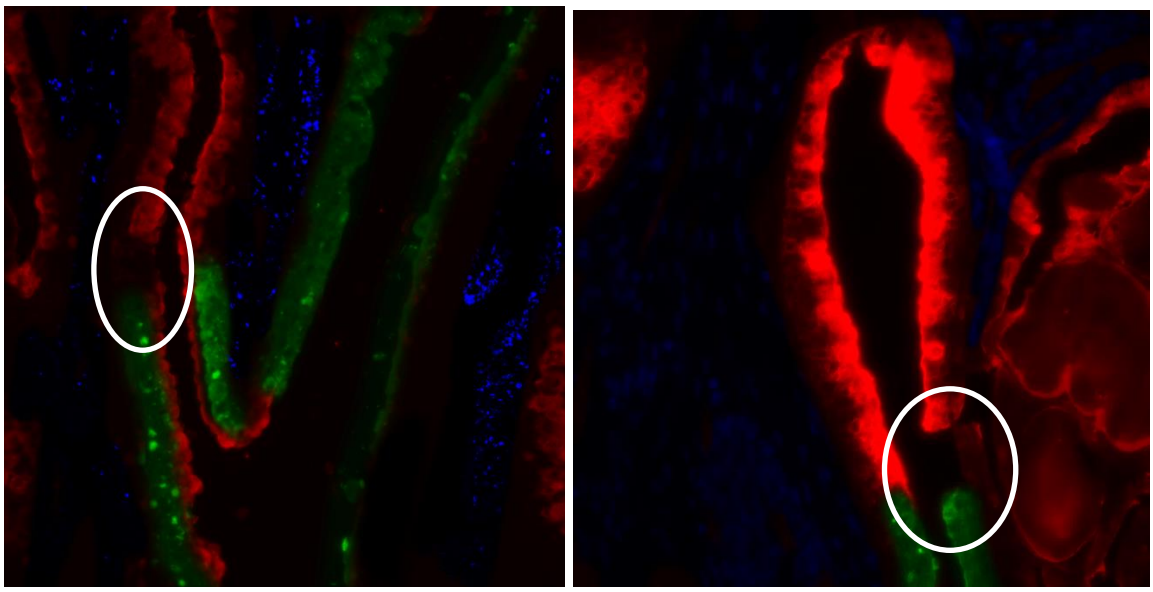


Figure 3.15 - There are certain spots in some of these sections (section 13891-2 and 13891-14 respectively shown here) where PSA and UPK (red and green respectively) are not taken up - potentially indicating the presence of cells not positive for either of them – possibly could be stem cells or maybe other types of cells as discussed below.

3.5. Limitations of the Opal Kit

In addition to heat stability, there were also two other issues encountered with the use of the Opal kit.

1. Photobleaching – Where exposure to light passing through certain filters even for extremely short amounts of time causes the fluorophores to be quenched leading to an inability to analyse the slide.
2. The apparent ‘mixing’ of colours from the fluorophore leading to complex images showing the existence of PSA and UPK on the same set of cells which is not possible at all – as evidenced by IHC optimisation.

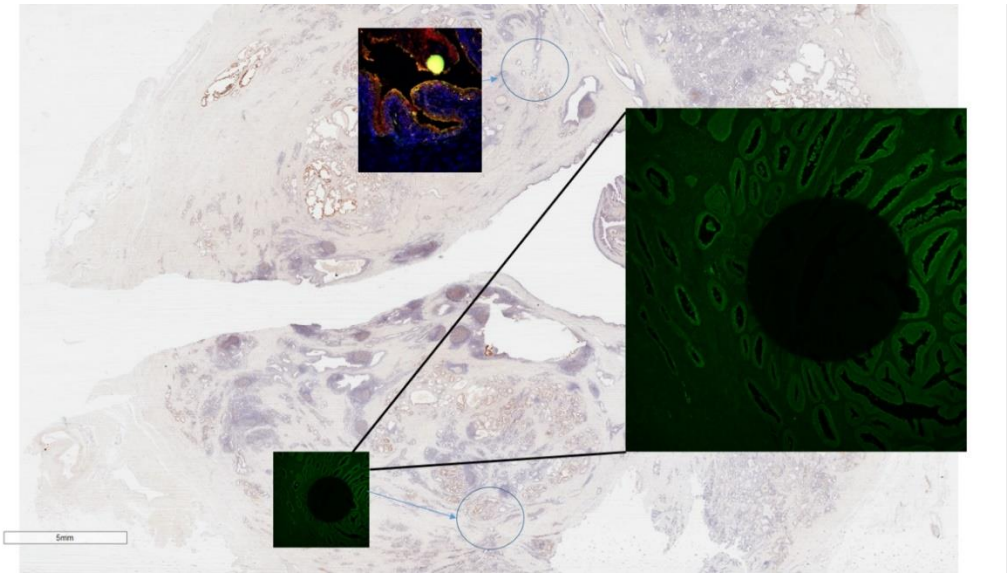
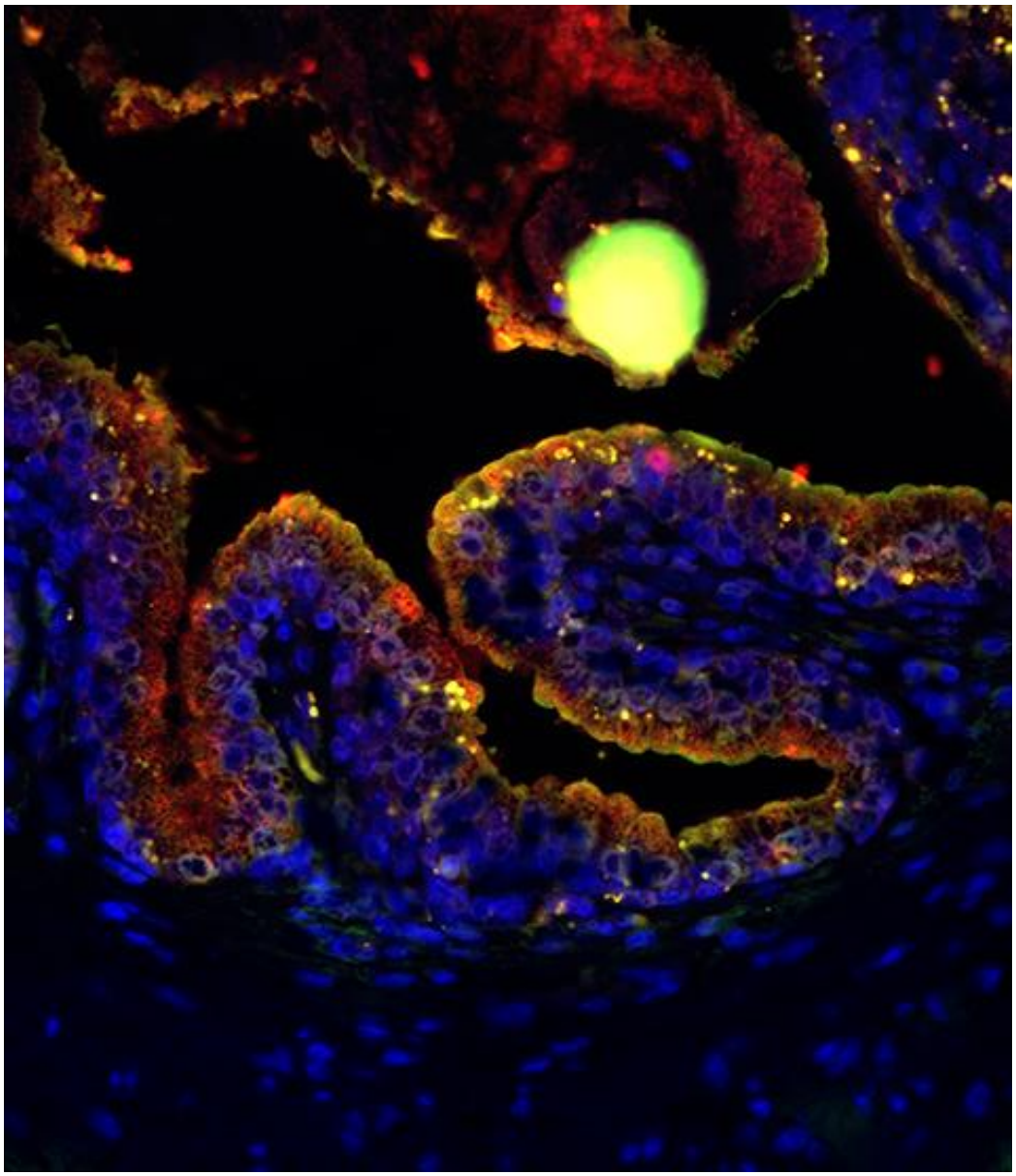


Figure 3.16 – Image from a patient's prostate tissue sample showing areas that show photobleaching (R) and an overlap of markers (L)



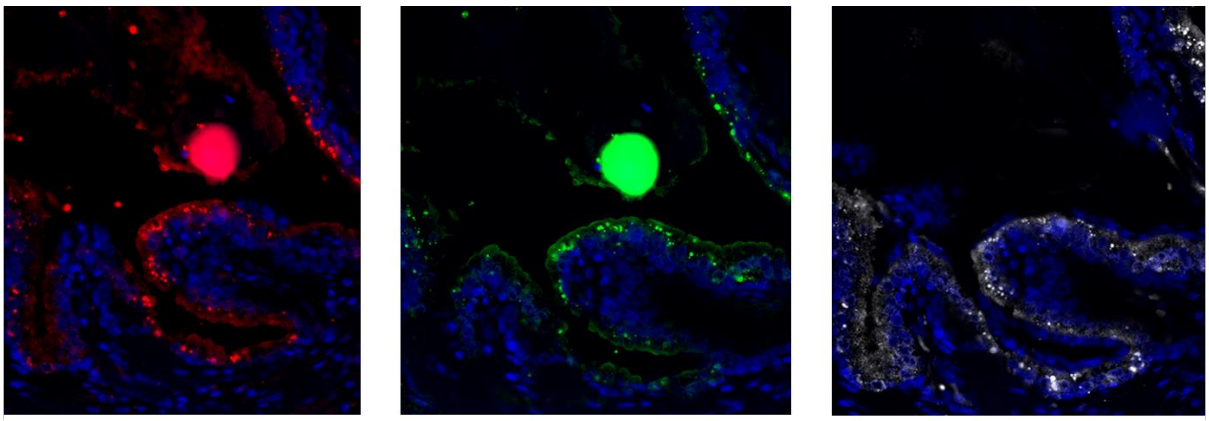


Figure 3.17 - Shown above is a complex image of a multiplex involving three different markers – PSA (red), Uroplakin 1b (green) and 34BetaE12 (white). The order of staining these markers was in the same order as mentioned in the previous sentence with PSA going first, UPK going second and 34BetaE12 going third. The complex image above shows considerable overlap of regions stained by PSA and UPK – something that is not possible.

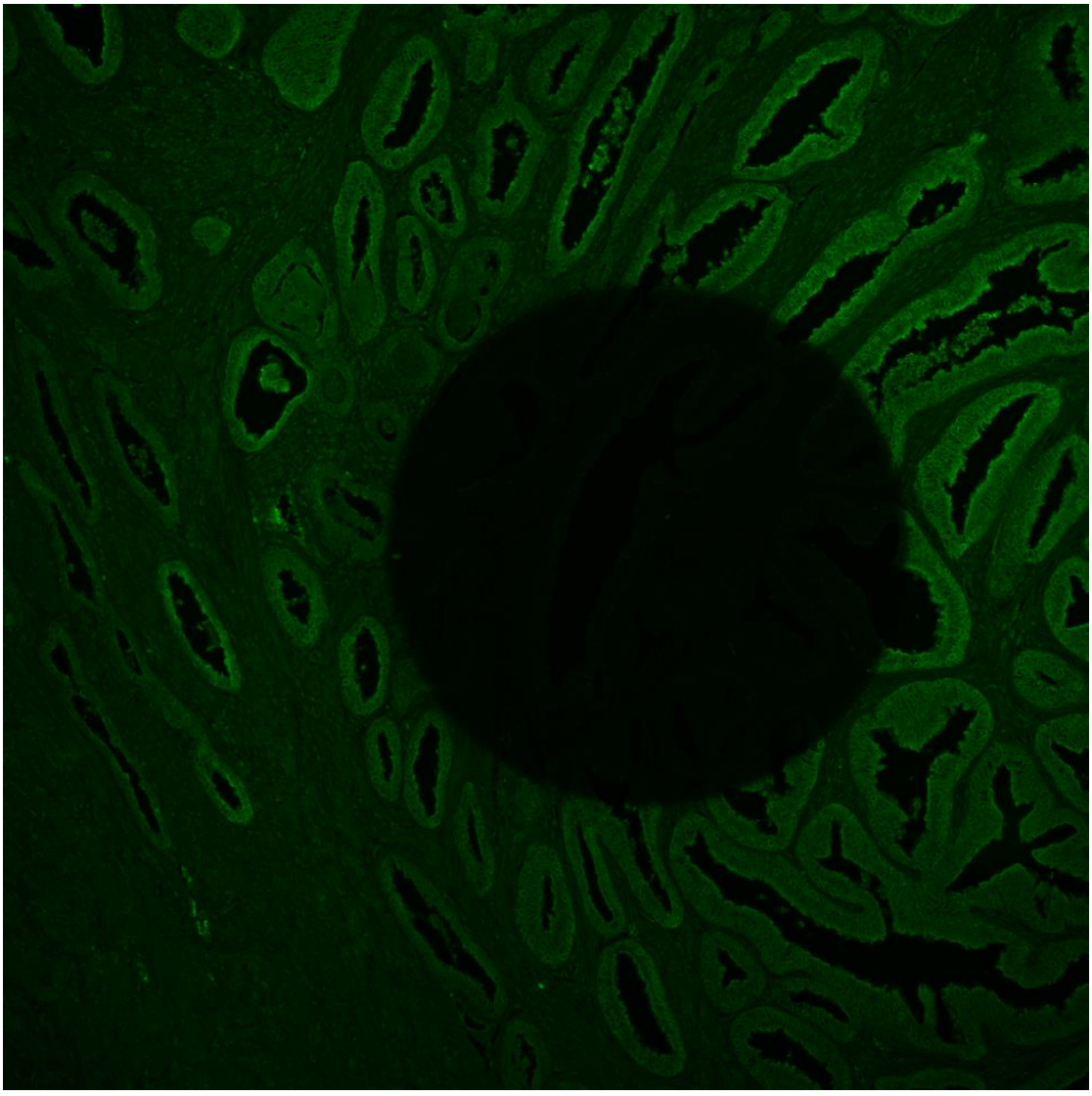


Figure 3.18 - Photobleaching after just 30 seconds of exposure to a particular filter on the Leica DM6

3.6. DAB-SIMPLE

Sequential Immunoperoxidase Labelling and Erasing (SIMPLE) described in section 2.5 is a method to visualise multiple markers on the same slide after sequential IHC first described by Glass et al. in 2009. An example of SIMPLE at work is demonstrated in their paper with work done on mouse cerebellum as shown in the figure below adapted from their paper.

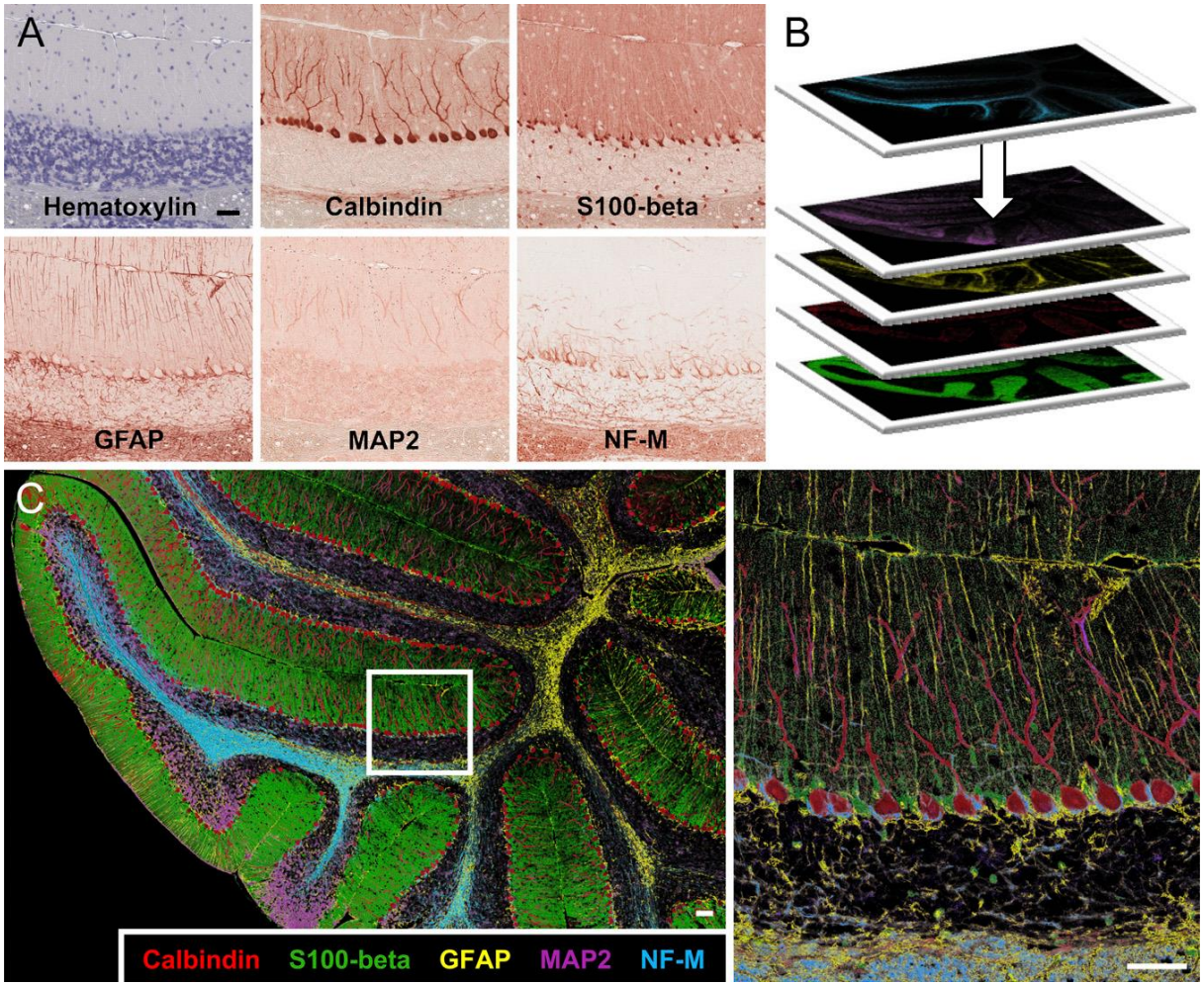


Figure 3.19 - Simultaneous visualization of five antigens in mouse cerebellum. (A) Adult mouse brain was counterstained with hemotoxylin, then sequentially probed with polyclonal antibodies to calbindin, S100-b, and GFAP, and monoclonal antibodies to MAP2 (AP18) and neurofilament (NF-M) 2H3. (B) The images were individually pseudocolored and overlaid. (C) The small boxed area in the left panel is shown magnified at right. The resultant image reveals the morphology of different cell types and fine details of interactions of Purkinje cells, Bergmann glia, astrocytes, and basket cell terminals that would not be obvious with single or dual labeling. Bar 5 50 mm. Adapted from Glass et al., 2009.

As seen in the figure above, the technique allows for the visualisation of multiple markers on the same slide which would be important in this study. Thus the use of

SIMPLE and its subsequent derivative of DAB-SIMPLE was an alternative to the Opal kit which had its limitations.

An in-house evolution of Sequential Immunoperoxidase Labelling and Erasing (SIMPLE), DAB-SIMPLE involves the use of DAB-IHC and Photoshop to generate compound images after multiple rounds of IHC. In the compound image generated below, PSA and UPK have been stained in consecutive, identical processes of DAB-based IHC, separately imaged, then combined together with a change of colour for each marker. In this case the PSA is coloured purple while the UPK is coloured green – surrounding the urethral lumen. This is an example of a region where the urothelium and the prostate epithelium are in close proximity to one another – hence the slide showing positivity for both UPK-1 and PSA. As we go further away from this region, there would be PSA positivity but no UPK-1 positivity as the tissue stained would then be purely prostate glands.

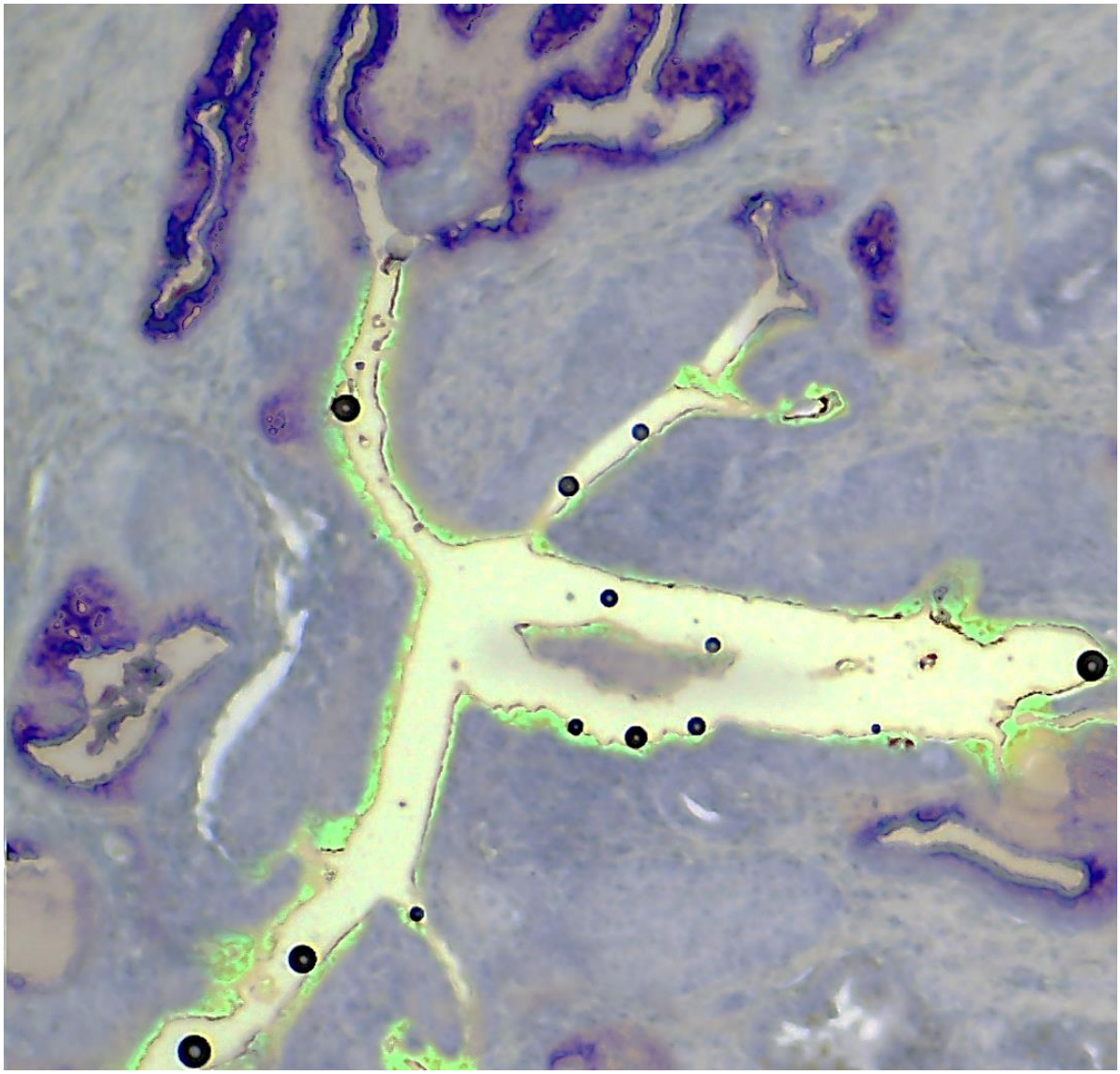
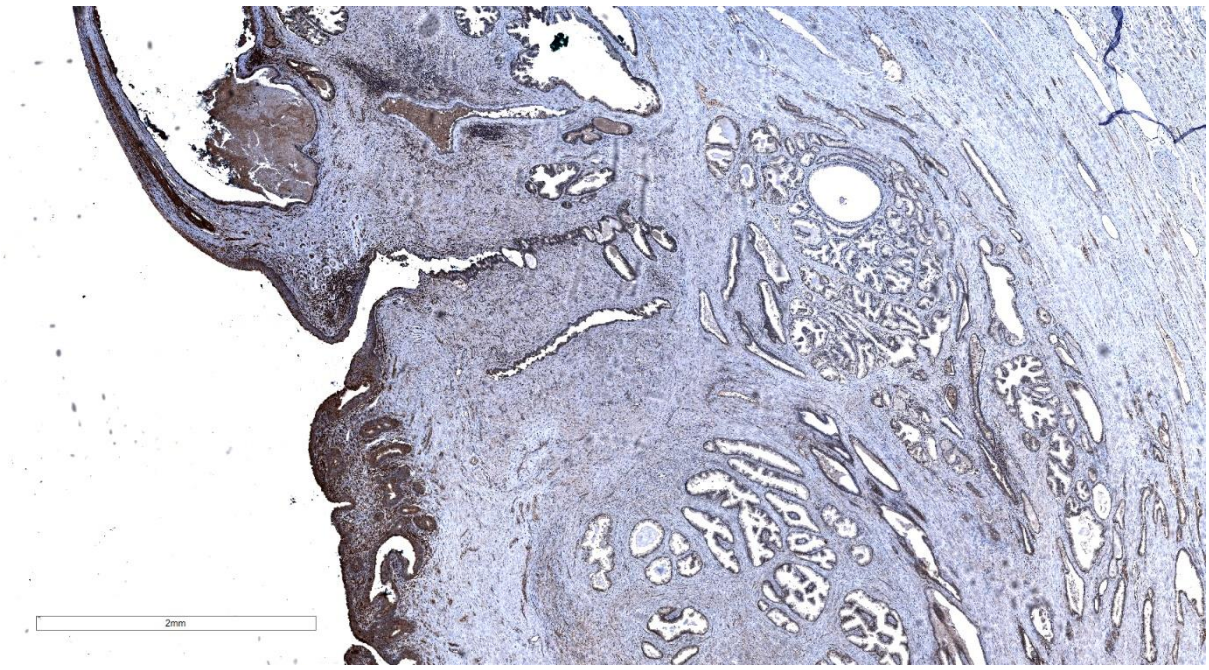
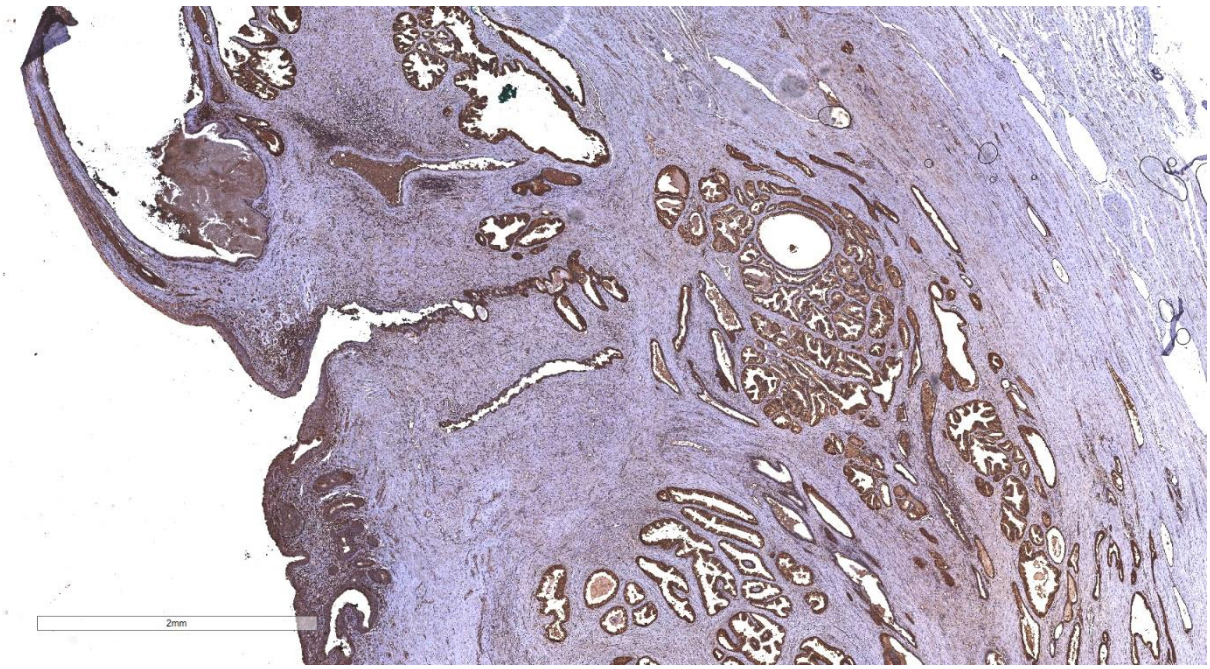


Figure 3.20 – A DAB-SIMPLE image of PSA and UPK1b

As a further proof of concept, these examples show a tissue slice with both prostate and urothelial tissue to demonstrate that with two rounds of staining with DAB this is possible.



(A)



(B)

Figure 3.21 – A) Slide with only UPK stained (arrow pointing towards the stained area) B) Slide with PSA stained after the staining of UPK. This shows that with multiple rounds of staining, and imaging

between each stain, it is possible to look at UPK and PSA and regions where they may be in close proximity to each other

The term DAB-SIMPLE was coined to include this form of multiplex IHC where some of the concepts of SIMPLE including imaging after every stage and staining of one marker at a time were combined with DAB-based IHC.

3.7. Multiplex IHC with using two different markers

Because of some inherent problems that come with the use of Photoshop – which is required in DAB-SIMPLE experiments – that have been discussed further below in Section 4.6, there was a need to change methodologies and explore the use of multiplex IHC. Multiplex IHC, as explained above in section 2.7, is a procedure which follows a protocol similar to standard IHC for a single marker but is used to stain multiple markers.

Before going into the procedure of doing immunohistochemistry for multiple markers, it was important to identify which other staining agent could be used to detect them. It was also important to note which ones could be used with DAB.

Since I was using the Vector ImmPACT DAB solution for detecting one substrate, I needed to know which was the other. For double labelling, Vector Labs have put out a chart indicating enzyme substrate compatibility and to be used as a guide to determine which of these were to be used with which other solution.

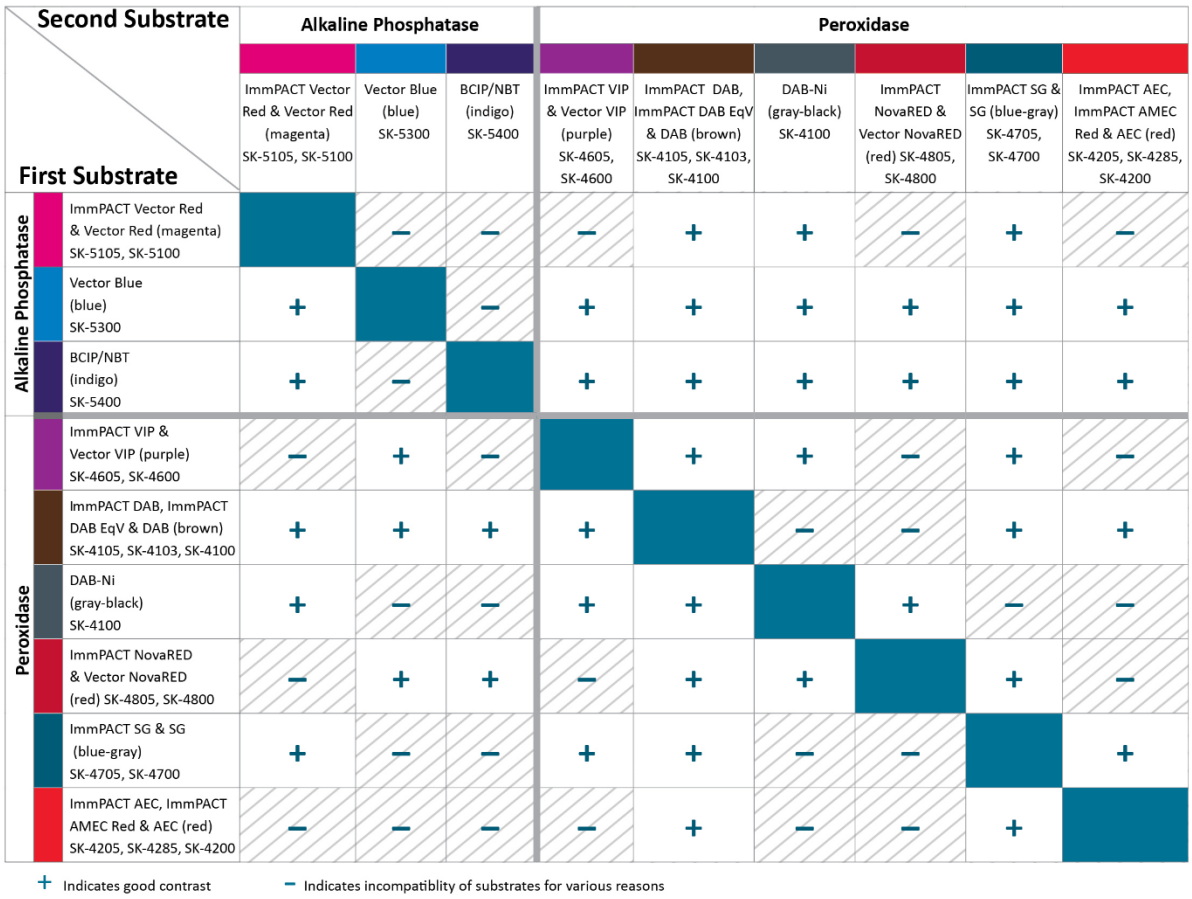


Figure 3.22 - List of second substrate versus first substrate compatibility as published by Vector Laboratories.

Now because DAB was stable through both heat and alcohol and it seemed quite prudent to subject the slides to a second round of decloaker mediated heating for antigen retrieval in order to further loosen any formalin linkages that may exist on the FFPE slide, DAB had to be used as the first substrate. The second substrate would need to be decided based on the chart above and based on certain other factors.

Based on the data above, the substrates that can be used second after DAB has been used as the first substrate are as follows and the reasons why some have been chosen and others have not have been discussed as well.

- ImmPACT Vector Red and Vector Red Magenta need permanent mounting – which would be a problem if more antigens need to be stained for as the same slide cannot then be used repeatedly – especially considering that at some stage in the future a stem cell marker would also need to be stained with PSA and UPK
- Vector Blue and BCIP/NBT (indigo) while giving good contrast with DAB – run the risk of being too similar in colour to the haematoxylin that is used to stain

nuclei and while that is a minor negative in comparison to the clarity of the image that could potentially be obtained – it could turn out to be fairly significant negative as well if the tissue is not stained properly. Moreover, some of the images on the catalogue given by Vector looked overstained. While this may be a problem in the staining process itself, it could also be a result of the substrate being overreactive.

- On the other hand, ImmPACT VIP and Vector VIP stain purple which runs the risk of being too similar to background eosin staining. It also requires permanent mounting only which has a similar problem as before.
- ImmPACT SG and SG (blue-gray) require permanent mounting as aqueous mounting can cause hardening of the tissue and may affect tissue architecture.

This left me with the only reasonable alternatives being ImmPACT AEC and ImmPACT AMEC. The two are very similar to each other in giving a red colour and also have the added advantage of the fact that a well established procedure (SIMPLE) has used AEC. Out of the two, AMEC Red was preferred because it was advertised to give more of a contrast to the DAB stain than AEC.

As a follow-up to the experiments above, multiplex IHC was done with two different substrates for PSA and UPK was done. The reason for just PSA and UPK is discussed further on in this thesis but the main reason is that these two markers help demarcate the main tissues involved at the stem cell niche as well as they are mutually exclusive. Multiplex IHC involved repeating the procedure of IHC and imaging at the end of it, unlike with DAB-SIMPLE where imaging had to be done at each stage.

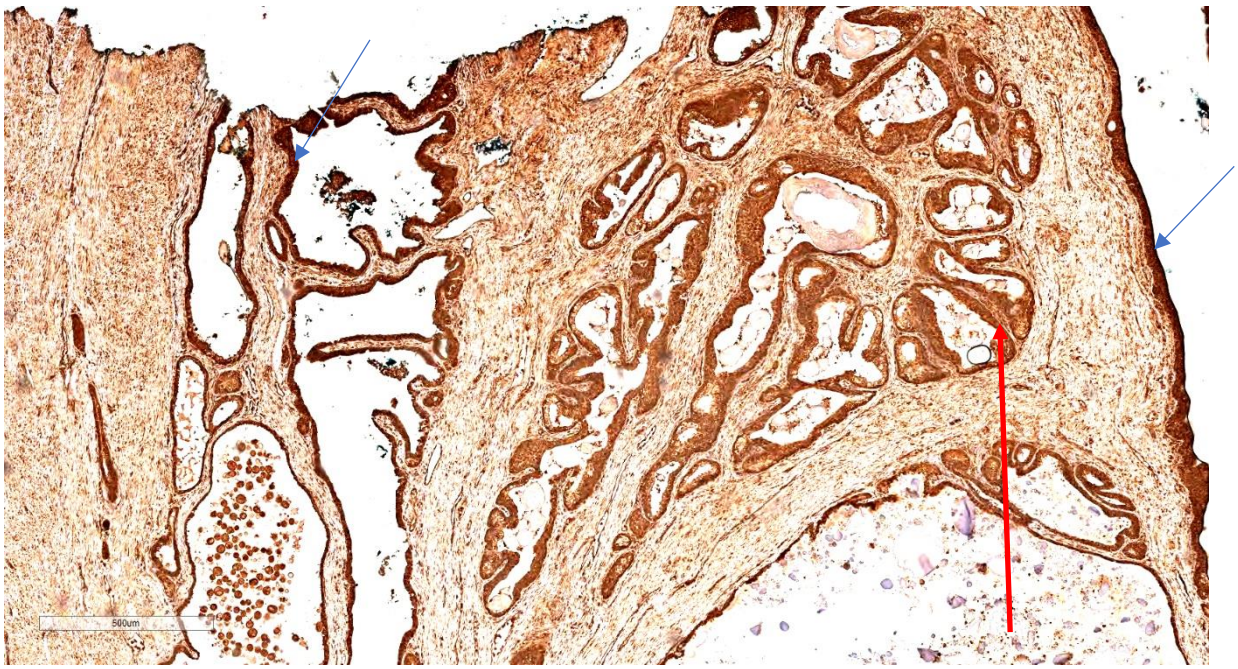


Figure 3.23 -The blue arrows point to the brown DAB stain that was used to stain UPK 1b while the red arrow points to the AMEC Red used to stain PSA.

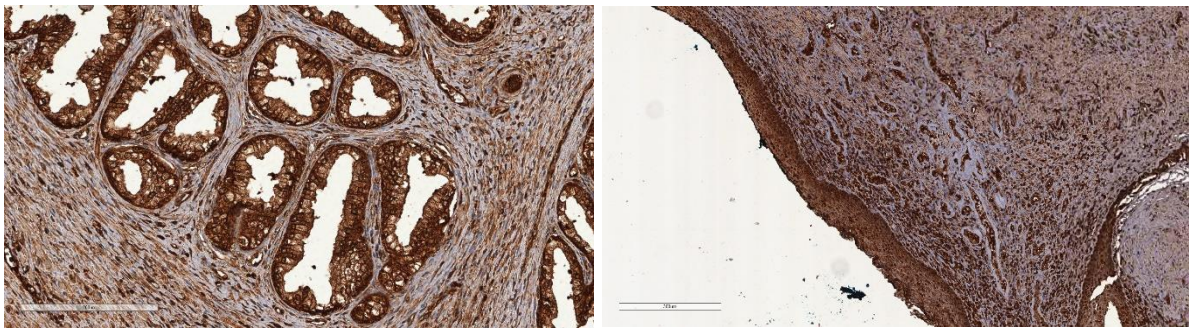


Figure 3.24 - Comparison of AMEC Red staining of PSA (L) on some slides with DAB staining of UPK (R) on the same slide showing how similar the staining looks

Furthermore, the use of DAB and AMEC-Red makes the parts of tissue stained by both these markers appear similar as can be seen below. However, on conversion using photoshop to an IF-like image on a dark background, it can be seen that the two colours are different as seen in Figure 3.21. More on this is talked about in Discussion.

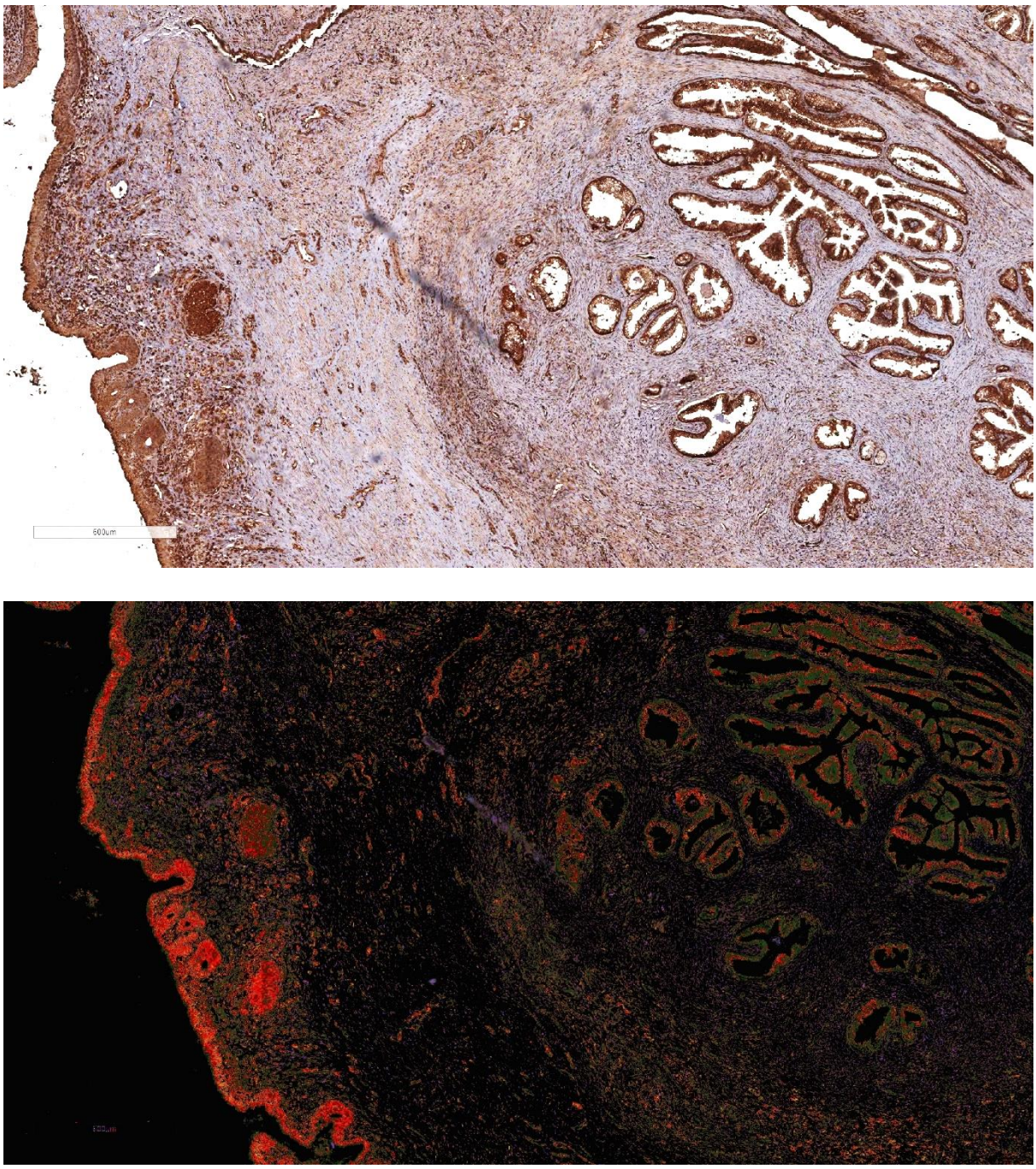


Figure 3.25 - However, despite looking similar in the first picture, DAB and AMEC-Red are actually different colours – something that can be confirmed by using Adobe Photoshop to replace colours. The brown of DAB stained UPK and urothelium is replaced by red and the red of AMEC-Red has been replaced by green. These however are not perfect as is discussed further below.

Chapter 4: Discussion

With Moad and his group's work describing the existence of prostate stem cell within a niche at the areas where the prostate epithelium and the urothelium meet in an interdigitating fashion, it was then the task to identify what cells made up the niche and how the niche was structurally as well as trying to elucidate the functional aspects of the niche including various pathways. However, understanding of the functional aspects requires a primary, pre-requisite understanding of the cells and tissues that make up the prostate stem cell niche – a field in which our knowledge was still in its infancy.

While performing IHC, serial dilutions were used to determine the ideal concentrations of each antibody and each marker and noted down. The concentrations determined through this method not only ensured that there was no overstaining and understaining in the slides but it also can be carried over to the Opal kit because as per the manufacturer's recommendation, the concentration to be used when using the kit is the same as the concentration that was found through serial dilution with DAB-based IHC.

All the markers shown in the table in Results were taken through to the Opal monoplex stage to optimise them with the Opal protocol. The idea was to test whether the concentrations that were deemed to be ideal to the DAB-IHC method would still hold up as the ideal concentration to use with the Opal reagents.

The ultimate choice of markers would come down, as mentioned before, to demarcating the different tissue types that are present in and around the stem cell niche. Each of these markers must be both specific to that tissue type and mutually exclusive from the other markers. The tissues that need to be marked out include the prostate epithelium, the urothelium and the stem cells themselves. In the context of prostate biology, this would be important because this would then offer definitive proof of the existence of stem cells and a stem cell niche within the prostate.

While other methods such as single cell sequencing can also demonstrate the existence of stem cells, it is important to note that the aim of this study is to look at the stem cell niche from a structural point of view to get an architectural perspective on its location within the prostate. Hence imaging techniques including multiplex imaging were the best way forward to visualise the stem cell niche within the prostate.

4.1. Opal Kit Monoplex

As mentioned previously, the Opal kit is a method to detect multiple antigens on an FFPE slide. The main difference between DAB-IHC and the Opal kit is the fact that the Opal protocol makes use microwave heat- mediated antigen retrieval as opposed to steam. In theory, microwave heat following detection of one marker allows for removal of primary and secondary antibodies, removal of any non-specific staining and reduction of tissue auto-fluorescence. However, the method did have other issues which shall be discussed further below.

While doing the monoplex for the Opal kit with each of the markers in the table above, it was established that the same concentrations that worked for DAB-based IHC would also work optimally for the Opal kit. NKX3.1 also responded better to the Opal kit and marked out luminal nuclear cells clearly. The other markers also worked well. P63 on the other hand was a marker that did not respond well at all to the Opal protocol. It was non-specific. The only concern with DLK-1 which seemed a little more specific with the DAB-IHC protocol than with the Opal protocol. However, it was still carried forward to the Opal multiplex experiments.

With DAB-IHC, DLK-1 staining seemed targeted toward very specific cells as can be seen in Figure 3.1.G. While actual quantification was not done, it seemed purely on visual evidence to be far less in number than when compared with staining seen on the Opal monoplex optimisation – seen in Figure 3.9 on the right-hand side. If quantification needed to be done, the abundance of DLK-1 positivity could be checked using specific software including ImageJ which has been used to quantify FFPE slides stained for different markers using the IHC protocol.

4.2. Opal Multiplex

Many multiplex experiments were carried out with different combinations of markers – not all of which are shown in the results section. The results of these experiments were twofold. On the one hand the Opal multiplex experiments performed after much modification gave rise to some insights towards the architecture of the stem cell niche but on the other hand it also revealed many problems with the method itself – which are addressed below.

Carrying out multiplex experiments allowed us to determine the right combination of markers in order to define the stem cell niche. At the very outset, it was determined

that a prostate basal marker, a prostate luminal marker, a stem cell marker and a marker for urothelial tissue were prerequisites. This automatically meant that every experiment of the Opal multiplex had uroplakin 1b in it. The choice for prostate luminal marker eventually came down to PSA and CK8+18. However, because cytokeratins 8+18 marked out both urothelial and prostate tissue, it was decided to go with PSA which would be an exclusive marker of the prostate luminal tissue. The mutual exclusivity of PSA and UPK would also help later on as shall be discussed further. With DLK-1 being the only stem cell marker, it was automatically included into the list of markers. Ki-67 was eliminated because while it marked out proliferative cells in general, these included both stem cells and non-stem cells which displayed proliferative characteristics. That meant that it would not be of much use to demarcate just the stem cell niche. When it came to basal cell markers for the prostate, there were two options. One was p63 and the other was 34BetaE12. p63 did not quite work very well with the Opal monoplex which meant that it had to be eliminated then and there leaving 34BetaE12 as the only other one. However, the disadvantage that I had to work around with this particular marker was that it also marked out urothelial tissue. The only difference between the two – as mentioned before – was that it marked out only the basal layer in the prostate while marking out both superficial and deep layers of the urothelium. Some of the early conclusions from these experiments thus allowed for elimination of certain markers.

The combination of different markers also led to the discovery of other issues that affected the use of this method.

4.2.1 Heat Stability

One of the first problems that I came across while doing multiplex experiments was that on performing the experiments, some of the markers would just not appear on the final multiplex even though the marker itself worked well on monoplex optimisation. Figure 3.10 is one such example.

The Opal protocol requires that markers be subjected to multiple heat cycles using a microwave when they are first in the combination that is to be tried. For example, in the procedure used to generate Figure 3.10, the order in which the markers were stained was as follows:

Table 4 – Combination of markers used in an experiment for the Opal protocol

No.	Marker stained for	Concentration	Opal Fluorophore used
1	PSA	1:5000	570
2	34BetaE12	1:100	620
3	Uroplakin 1b	1:20	650

This was followed by DAPI staining and all the Opal fluorophores were stained with a concentration of 1:100 which was found during monoplex optimisation to be ideal to visualise markers.

In the complex image that is generated after all the markers have been stained and visualised, it is clear to see that PSA and Uroplakin 1b have both shown up in the final image but 34BetaE12 has not shown up. This despite there being no issues with 34BetaE12 staining during monoplex optimisation. The observation was further validated in another early multiplex experiment performed with the following combination and order of markers.

Table 5- Combination of markers used to stain tissue in the Opal protocol for a second experiment

No.	Marker stained for	Concentration	Opal Fluorophore used
1	PSA	1:5000	540
2	NKX3.1	1:1000	570
3	34BetaE12	1:100	620
4	CK8+18	1:100	650
5	DLK-1	1:100	690

Once again, this was followed by staining with DAPI and all the Opal fluorophores were stained with a concentration of 1:100.

As seen in the image below, the complex image appears to show PSA – which has survived four subsequent rounds of antigen retrieval with microwave heat. Also shown in the image is NKX3.1 – but only partially as some cells have stained for the nuclear marker while others have not. However, as confirmed by individual imaging (not shown here), 34BetaE12 which marks out the basal layer of the prostate is nowhere to be seen. DLK-1 being the last marker added shows up – although based on the pictures it cannot be determined if it has marked the right cells with complete clarity. CK8+18

turned out to be a redundant marker in a certain sense as it overlapped with the regions stained by PSA.

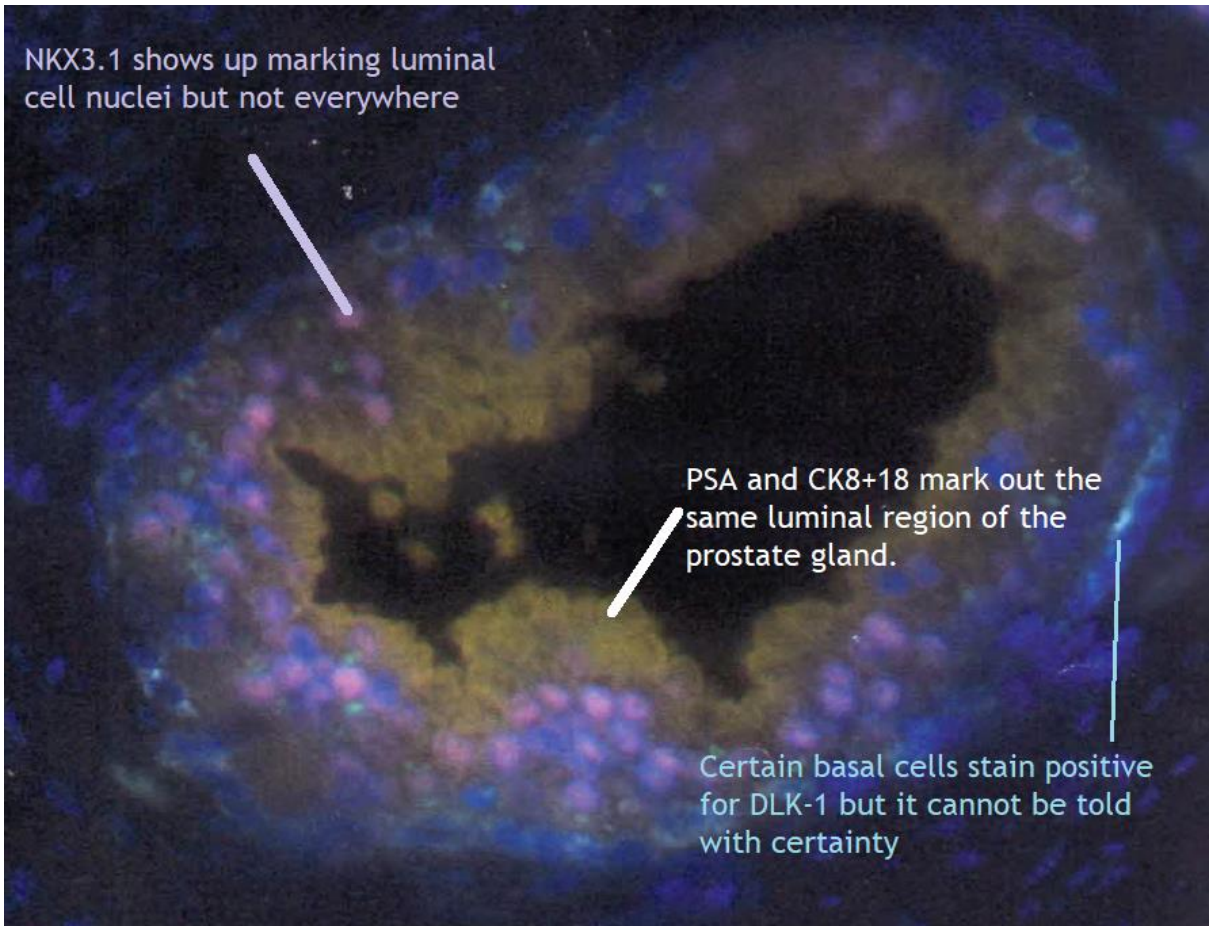


Figure 4.1 – The composite image from one of the first multiplex experiments whose markers are listed in Table 5.

Furthermore, as seen in this image, the light filters on the Leica DM6 would sometimes allow for visualisation of multiple markers as the emission wavelengths displayed on the microscope

Overall this begs the question as to why certain markers are more stable in the heat as opposed to others. For example, through multiple such experiments, it was found that PSA and Uroplakin could withstand up to five cycles of microwave-heat mediated antigen retrieval but other markers like DLK-1 and 34BetaE12 could not withstand even one cycle of heat.

At this stage, it is important to go back and look at the nature of the relationship between antigen retrieval and Fixed Formalin Paraffin-embedded tissue (FFPE tissue). Shi et al. published the first known article on antigen retrieval in 1991. It was a rudimentary method which involved just boiling the tissue in water – but it was a step

that would significantly impact IHC procedures in a positive way. Since then it has been established that in order to break through cross-linkages caused by formalin, it was necessary to subject the FFPE tissue to high temperatures. Higher temperature AR yield better results with IHC as compared to lower temperatures. If indeed a lower temperature is used, to achieve the same intensity of AR-IHC it would need to be used for a longer period of time.

With the Opal kit, the microwave mediated heat would be ideal to break through formalin cross linkages. A few experiments (not shown here) were performed to see if changing the antigen retrieval method would make a difference to the image that is generated. However, no significant differences were found and hence it was decided to continue with microwave heat.

However, there is one major issue that has come up with the use of the Opal kit specifically in relation to the use of the microwave heat through multiple cycles. And that is the problem of epitope denaturation.

Most antigens in the human body are proteins entirely or have some component that is a protein as part of them. The exceptions would be antigens such as those found on the surface of the red blood cells which could be sugars. However, the markers that we have chosen to look at in this project are by and large proteins.

In each antigen, there is a specific site to which the antibody binds – and that region is known as the epitope. The existence of the epitope is crucial as the physical structure (the primary, secondary and tertiary structure) of that epitope can only be bound to by a suitable antibody that complements the epitope. Exposure to extreme amounts of heat can tend to denature protein structures and with respect to the antigen epitopes, there exists a variation between different antigens as to the number of heat cycles that they can withstand. A study done by Lee et al. (2020) entitled “Multiplex immunofluorescence staining and image analysis assay for diffuse large B cell lymphoma” demonstrates the effect that multiple heat cycles can have on epitope stability with some fluorophores likely to show increased intensity of fluorescence and others potentially showing decreased intensity of fluorescence.

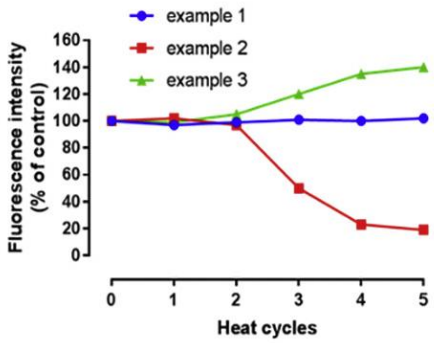


Figure 4.2 - Epitope stability is evaluated following several cycles for heat-mediated antibody stripping. Example 1 (blue) shows a stable level of fluorescence, and examples 2 and 3 show denaturation of epitope with decreased intensity and increased intensity due to continued epitope retrieval, respectively. Image taken from "Multiplex immunofluorescence staining and image analysis assay for diffuse large B cell lymphoma" (Lee et al., Journal of Immunological Methods, March 2020)

From the above data, it is clear that one of the effects of using microwave heat is epitope denaturation. Denaturation can occur at varying rates and with varying consequences. The study done above was not evaluating epitope stability as its primary aim hence there is only limited data. But that does help explain why some of the markers remain stable in my experiments through multiple cycles of microwave heat while others could not withstand even one more round of microwave heat.

4.3. Elimination of markers

Based off of the experiments with different combinations of markers, elimination of markers was undertaken throughout the process – continuously narrowing down for reasons as indicated by the table below.

Table 6 – Reasons for elimination of various markers to continue with a selected few.

Candidate Marker	Reason for Elimination
34BetaE12	**NOT ELIMINATED***
p63	Not heat stable through 1 cycle of heat
Androgen Receptor	Did not stain well with the Opal kit
Prostate Specific Antigen	**NOT ELIMINATED**

CK8+18	Covers the same tissue as PSA plus is more non-specific as it also stains the urothelium as well
NKX3.1	Only partially heat stable and plus with the existence of PSA as a stable luminal cell marker, it did not make sense to go forward with NKX3.1
Ki-67	Marks out only proliferative cells and hence is not specific to stem cells.
DLK-1	**NOT ELIMINATED**
Uroplakin	**NOT ELIMINATED**

4.4. Experiments with 34BetaE12, DLK-1, PSA and UPK-1b

Based on the paper by Moad et al., the stem cell niche is located at the intersection of urothelial tissue and prostate epithelium. This would mean that in order to define the stem cell niche across various sections of the slide, there exists a basic necessity to define the following

i. Stem cells
ii. The prostate epithelium – could be defined by marking out the basal layer and the luminal layer
iii. The urothelial tissue

The issue of epitope denaturation did creep up again during early experiments performed with the four markers. Both 34BetaE12 and DLK-1 could not withstand more than one cycle of microwave heat. This posed a problem in terms of the fact that while PSA and UPK can be stained in whatever order as they are both stable, only one of either 34BetaE12 or DLK-1 could be used. Adjusting various settings during the experiment including the power of the microwave and even trying out steam-based antigen retrieval using a decloaker did not change this particular effect.

Moreover, the antibody used for DLK-1 did not take particularly well to the Opal protocol giving very inconsistent results. Hence a decision was made to drop this

antibody temporarily. This would leave me with UPK, PSA and 34BetaE12 as markers to look for.

With Moad et al. providing us with a picture whereby the prostate epithelium and the urothelium interdigitate and amid these interdigitations lie the stem cell niches, it can be concluded that if we can map out regions with markers that indicate the junction of the prostate epithelium and urothelium, there are bound to be segments within some of these regions that would in some way indicate the presence of stem cells. This could quite likely be a region that does not take up any staining. Dual staining of both PSA and UPK is unlikely but if it does show up – it does raise questions as to whether the stem cell has any role in urothelial tissue generation as well. There could also be regions where the two markers are taken up by the respective cells and there is a clear demarcation between the two but with no intervening regions that indicate the presence of stem cells. 34betaE12 might also exist but there is a potential problem in that it could overlap with the staining of the uroplakin.

However, before we could get that far we hit upon yet another problem when it came to just using these three markers.

The images in Figure 3.16 show regions within that section of tissue which are stained by both PSA and UPK – something that has been established through prior IHC experiments as being impossible. PSA stains prostate tissue without staining urothelial tissue and UPK stains urothelial tissue without staining prostate tissue. Hence tissue that looks yellow in the picture below – indicates a region that has stained for both uroplakin and PSA – further confirmed by the individual images taken for each of the markers which shows localisation of both PSA and UPK to the same areas in some places.

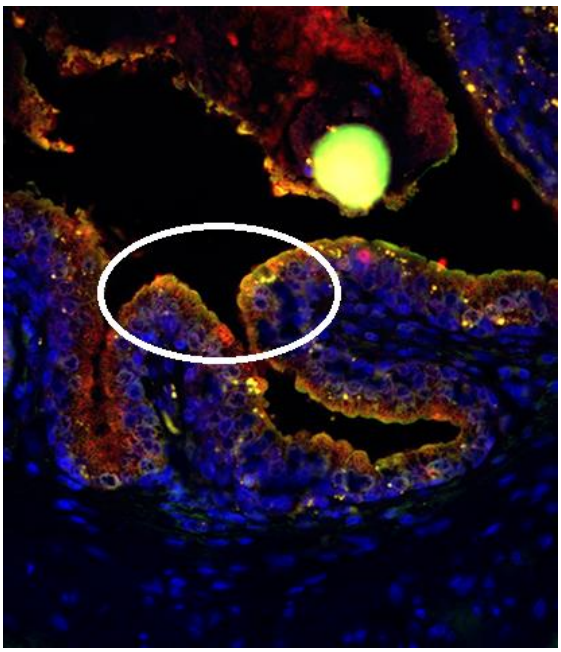


Figure 4.3 – The circled area shows areas showing an overlap of colours signifying localisation of PSA and UPK to the same areas

This can potentially be attributed to the fact that in some regions there is a close proximity between urothelial tissue and prostate tissue – even sometimes resulting in overlaps when looked at through a radial tissue section of the ducts as is the case with the tissue samples examined. Because the tyramide-fluorophore is activated through reaction with the HRP and forms covalent bonds with the tyrosine residues present in the near vicinity of the protein of interest following the binding to the specific protein of interest by the specific antibody and an HRP conjugated secondary antibody, there could be a lot of overlap of the binding of PSA and UPK in regions of close proximity between the two – such as the interdigitations amid which the stem cell niche is thought to be present.

Further as can be seen in the individual images from Figure 3.17 there is a considerable overlap between uroplakin and 34BetaE12 staining which can be explained by the regions the two markers stained – as discussed earlier.

Hence following this experiment and to get a clearer picture, it was decided to narrow down the marker list further to just two markers – PSA and uroplakin 1b – which are mutually exclusive. A decision was also made to try and find regions where the two may be in close proximity but not so close that the impact of Opal staining would result in the generation of images with colours that indicate that PSA and UPK bind together.

Furthermore, it was also found that the Leica DM6 microscope which was being used had certain settings which needed to be changed when switching between viewing PSA staining and viewing UPK staining. However, when allowing for automated image capture some settings are locked together, making it a compromise on both settings and hence that compromises the image quality. To get around this issue, I decided to do individual image captures of PSA and UPK and then combine it using Adobe Photoshop 2020 and using their merge image feature.

This has resulted in the generation of images that were of value when it came to evaluating the border between urothelium and the prostate epithelium and the possible existence of stem cell niches.

The image in Figure 3.12 is an example that was obtained after staining with just uroplakin and PSA and following the modification of the image capture process on the Leica DM6. In there it is clear to see that in some regions of the tissue sections, there exists a relationship between PSA and UPK whereby the PSA seems to be luminal and the UPK seems to overlap over PSA. Further looks into this relationship can be done by staining slides sequentially and seeing whether the border between the urothelium and prostate epithelium seems to display any change in characteristics. To this effect, a further few experiments were done with the staining of slides that were 16µm apart. The results are shown in Figure 3.13.

As indicated by the white line, there is the presence of a distinct boundary and a distinct location for the overlap of prostate and urothelial tissue. 16µm spans four sequential sections of tissue. And by the time we have gone 32µm deeper into the section of tissue we can clearly see that the prostate gland and the urothelial tissue are two separate entities, indicating that there is some sort of an overlap between the two for a very short depth into the tissue.

The image does lead to questions about whether or not there is an influence of one tissue over the origin or functioning of the other. These questions need to be answered further through functional studies of the urothelium and prostate – as explained later.

Shown in Figure 3.14 is another region with similar characteristics - where what seems to be just a region of urothelial tissue also shows the interaction between urothelial and

prostate tissue. From the two images it is clear to see that there is some sort of overlap between the urothelial tissue and prostate tissue.

I would also like to draw attention to two specific images in Figure 3.14. In both of these images, there exist regions between the urothelium and prostate epithelium where neither UPK nor PSA are taken up. This could indicate one of two things.

1. That it is nothing more than an artefact of staining and it is quite possible that there are normal prostate or urothelial cells whose epitopes haven't been tagged by the fluorophore for example
2. The other possibility is that there are actually cells there that exist and do not take up either antibody (and subsequently fluorophore) because they are fundamentally different from both these types of cells.

If the first point is true, the way to verify it would be to see if these "gaps" occur in the next sequential slides i.e., the slides that are 4µm apart. If they don't and the antibodies are taken up by cells, then it is quite likely to be an artefact. It does not rule out different types of cells being present in just that one section but it does make it significantly less likely.

If the second point is true, the way to verify it would be to verify it using a marker that tests for stem cells. In studies done by Moad et al., that marker would be DLK-1. However, the antibody that they used for their study is not being produced anymore – hence an alternate antibody for DLK-1 is needed. However, the DLK-1 antibody that I tested with did not seem to work convincingly well with the Opal kit even though on IHC with DAB it seemed to working fine and marking out cells that had DLK-1.

Another potential method to look at it would involve results from the paper published by Henry et al. (2018). As mentioned earlier, single cell RNA sequencing data showed the existence of two "other epithelia" (OE1 and OE2) in addition to the traditional classification of luminal, basal and neuroendocrine cells. Revisiting the locations where OE1 and OE2 were found (see table below) it is clear to see that club cells could potentially be stem cells.

Table 7 – A table summarising the findings of the paper from Henry et al. describing the type and location of OE1 and OE2 within the prostate and urothelial tissue

Type Of Cell	Location

KRT5+/KRT14-/KRT13+ Hillock epithelial cells	Prostatic urethra and collecting ducts Central zone surrounding ejaculatory ducts
KRT5-/KRT8-/SCB1A1+ Club cells	Prostatic urethra and collecting ducts but rare in the prostate

The reason that this hypothesis is more favourable than hillock cells being possible stem cells is because based on studies done earlier by Moad et al., it is clear that the stem cell niche should exist close to the urethral area given the involvement of the urothelial tissue. While both club and hillock cells satisfy that requirement, the crucial difference is in their prevalence within the rest of the prostate.

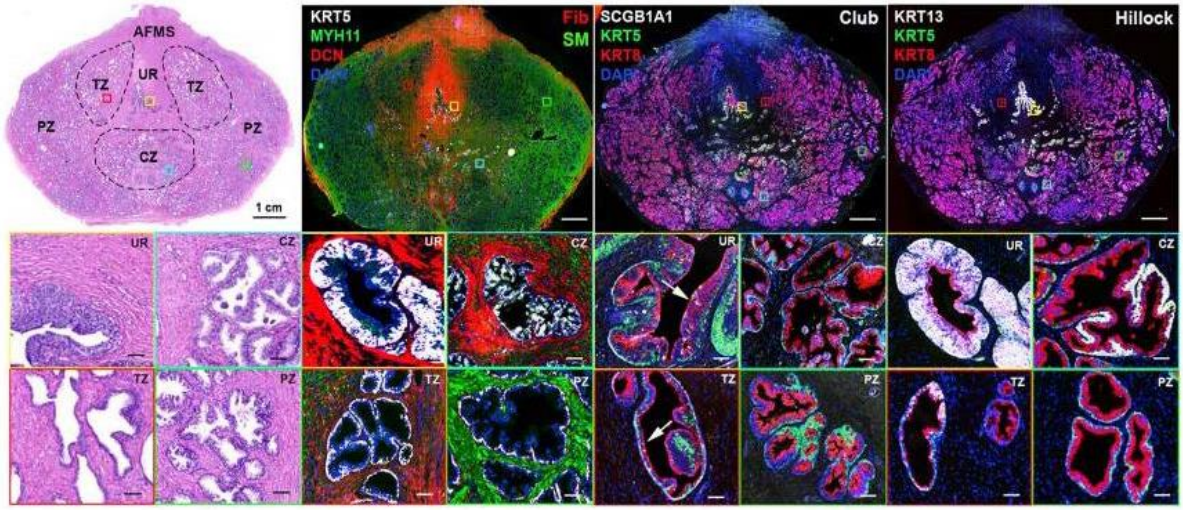


Figure 4.4 – Adapted from Henry et al. (2018) the images above show positivity for various markers of prostate cells in different regions of the prostate.

Stem cell niches and as a corollary, multipotent stem cells are not found very often within the prostate if at all – as seen from the images above – adapted from the paper by Henry et al. Hence from that point of view, the idea that KRT13+ hillock epithelial cells would potentially be stem cells is negated to a large degree because it is found in the central zone. However, there is still a chance that it could be the possible stem cells that are indicated by KRT13 positivity.

On the other hand, SCB1A1+ cells are rare in the prostate but present near the prostatic urethra and collecting ducts which would makes these cells the more logical candidate to the be the stem cells and these in turn could lead us to the stem cell niche.

However, in order to exactly determine which of the two is the actual stem cell or whether they are even the stem cell at all or just another type of cell which is not the stem cell is something that can only be done with experiments – both to determine structural and functional aspects of these cells. This is discussed further in Future Works.

4.5. Photobleaching

While the generation of the images above led to some understanding of the prostate stem cell niche and the architecture associated with it, there was another problem with the use of the fluorescent microscope and the Opal kit. This was that of photobleaching.

Photobleaching (also termed fading) occurs when a fluorophore permanently loses the ability to fluoresce due to photon-induced chemical damage and covalent modification.

With the Opal kit and the images obtained through it, there is an extremely high level of photobleaching that occurs thanks to the cleavage of the covalent bonds formed between the fluorophore and the epitope it is bound to – causing a loss of signal. The loss of signal was so quick and so great that even within 30 seconds of exposure to a light filter of appropriate wavelength to visualise it, the signal was lost – as is seen in this image in Figure 3.18.

The above mentioned image shows a complete loss of signal when exposed to light of a particular wavelength used to visualise the Opal fluorophores. In the case the marker that was to be visualised was PSA in a region of tissue with just prostate glands. This is a problem with the Opal kit that necessitates extremely quick image capture – something that cannot be done easily.

Having such a limitation makes it very difficult to capture a compound image after adjusting settings for each marker because while adjusting settings for each marker on a filter of different wavelength, there is a loss of signal for each of them. This can compromise the compound image that is generated due to loss of signal. The loss of signal may also not be in the same amount further reducing the quality of the image as well as any arguments for its validity.

4.6. SIMPLE and DAB-SIMPLE

Sequential Immunoperoxidase Labelling and Erasing Method (SIMPLE Method) is a technique that was published by Glass et al., in 2009 in the Journal of Histochemistry

and Cytochemistry. The procedure and method for this has been discussed earlier in the Methods section of this thesis.

SIMPLE allows for repeated rounds of labelling of antigens with specific antibodies by using a rapid non-destructive method for antigen-antibody dissociation facilitated by the use of alcohol soluble peroxidase substrate 3-amino-9-ethylcarbazole (AEC).

While this method would have been ideal, due to a delay in arrival of the potassium permanganate (discussed further below in the section discussing the impact of COVID-19 and the lockdown from March 2019 to June 2019) that was needed for washing off the antibody, it led to me improvising and devising a method deriving a large portion of its principles based on SIMPLE and DAB-IHC.

One of the characteristics of DAB is that it is resistant to both heat and alcohol. This means that a slide could theoretically be stained multiple times with DAB for different markers using the same procedure as normal IHC without fear of loss of previously stained markers. And taking a slight inspiration from SIMPLE, imaging was done at each stage and Adobe Photoshop was used at the end to try and generate a combined image picture by using the 'replace colour' function to give each marker a distinct colour. This is seen in figure 3.20.

One question that needed to be solved early on was whether repeated exposure to heat using a decloaker was needed or whether one round was enough to break the formalin crosslinkages. In the end I decided to go with multiple rounds as I felt that tissue architecture was not being compromised by repeated exposure to heat for a limited number of times even with the Opal kit and hence the same thought process could apply here.

One trial run was done with just PSA and UPK and imaging was done after the staining of each marker just like in SIMPLE – a whole slide image using the Aperio Scanscope imaging system. Adobe Photoshop CC 2020 was then used to change the colour of both PSA and UPK and the "Merge Images" function was used to create a composite image with both markers. This allowed us to see a composite image with both markers represented and as discussed before, potentially allow us to see certain spaces that could suggest the existence of stem cell niches or stem cells.

In the compound image generated and shown in Figure 3.20, PSA and UPK have been stained in consecutive, identical processes of DAB-based IHC, separately imaged,

then combined together with a change of colour for each marker. In this case the PSA is coloured purple while the UPK is coloured green – surrounding the urethral lumen.

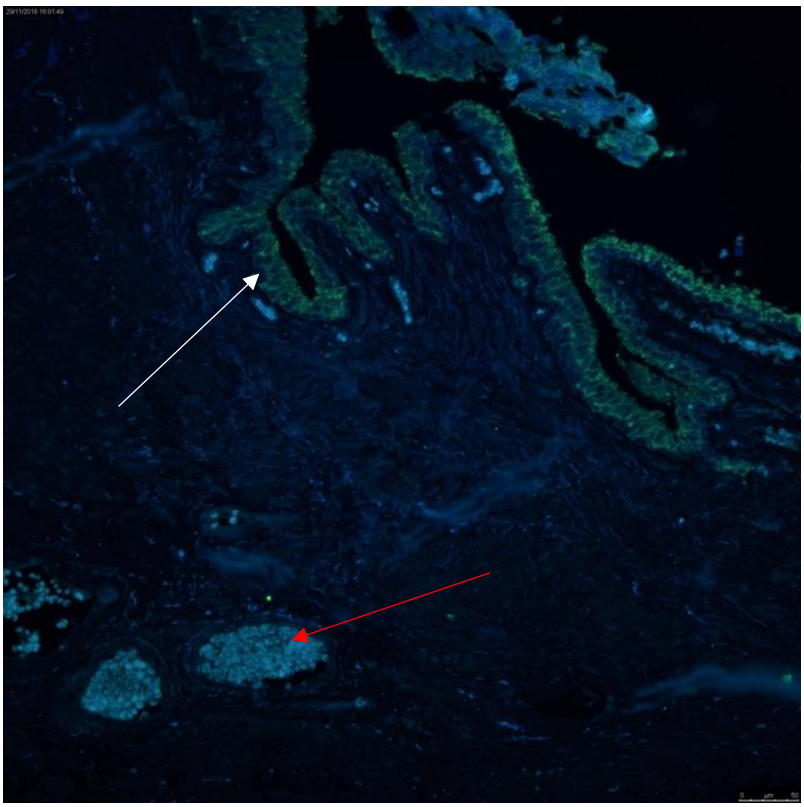
However, right off the bat, there are certain problems that are visible. One of the key issues here is that the “Replace Colour” tool on photoshop is that while it does a good job of replacing most of the primary shade of brown that appear in the vast majority of the marked tissue, it does not account for variations in the shades of brown and that becomes a problem with DAB-based IHC where sometimes even if it stains the right areas, the stain is not uniform in intensity throughout. This inconsistent pattern of staining can then lead to inconclusive deductions and interpretations of the image.

The other problem – which can be solved – is the fact that on merging the two images through photoshop, the background becomes less clear. However, this is just due to a lack of proper focus on the tissue during image capture and this can be rectified by focusing the image properly.

4.7. Limitations with Adobe Photoshop

One of the limitations of using Adobe Photoshop and the ‘replace colour’ and ‘merge image’ functions of the application was the inconsistency of replacing different shades of the same colour – which was discussed above. This would make interpretation of data very difficult. Moreover, the function can also stain artefacts.

For example, consider the following image taken from Opal monoplex optimisation of PSA and shown earlier in Figure 3.5.



In the above image, the white arrow points to a row of cells that have been marked for PSA – clearly visible with the green coloured appearance being luminal and on prostate cells. The red arrow shows an artefact that has appeared on the slide.

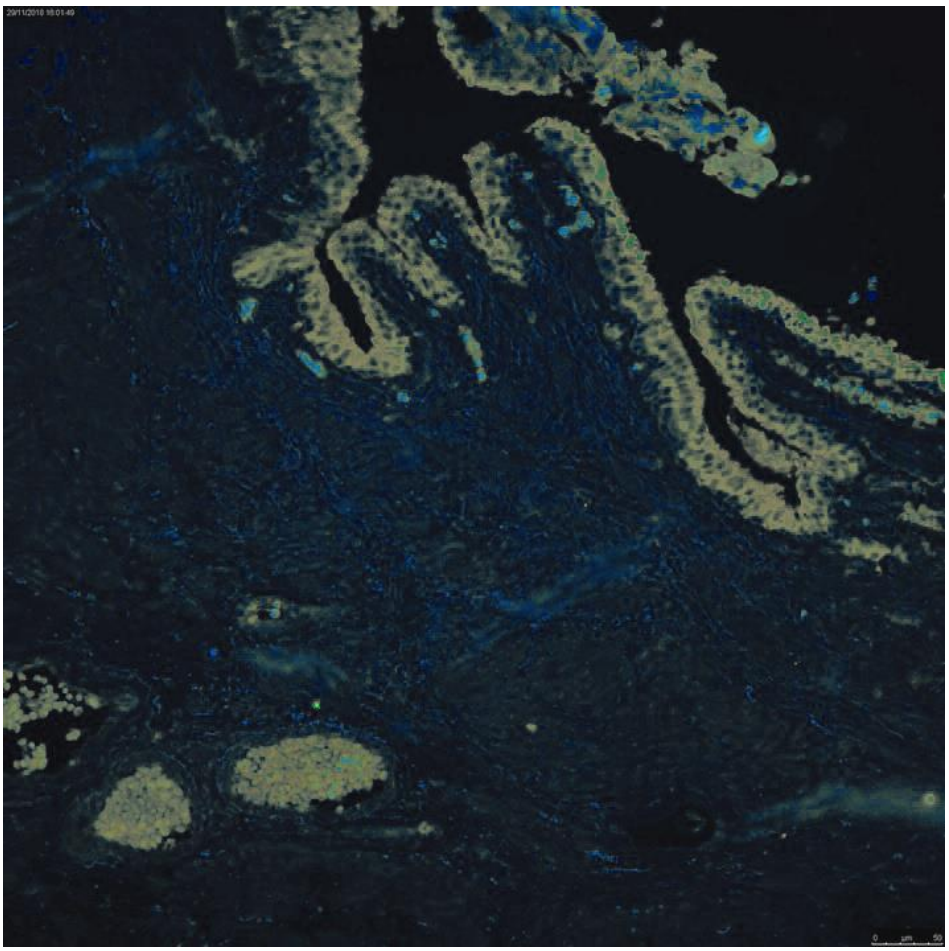


Figure 4.5 – Change to image in Figure 3.5 after the use of the Replace Colour function in Adobe Photoshop demonstrating the lack of recognition of difference between two colours.

Following the use of the replace colour function, it is clear to see that while it changes the colour of the PSA, it also changes the colour of the artefact. This change here is an obvious one but it can easily be conjectured that there may be certain unobvious errors like this that may happen – especially in the case of markers that only mark out very few cells in the first place like DLK-1.

This necessitated looking into alternative methods – one of which was Multiplex Immunohistochemistry (m-IHC). Since there were only two markers – PSA and UPK to look at, m-IHC would be a useful way of doing it as one of the reasons the Opal kit and SIMPLE was chosen was to allow for the use of more than 2 and upto 5 markers (6 including DAPI in the case of the Opal kit). With only two markers m-IHC could prove to be a much better tool to demarcate tissue. The other advantage is that both PSA and UPK have been optimised very well for IHC meaning their use could be easier with this method and the procedure is very simple and well established and standardised as well.

4.8. Multiplex IHC

Multiplex immunohistochemistry involved detecting multiple antigens with the same process of immunohistochemistry as used for DAB based staining but instead trying to use other staining agents as well.

Staining with AMEC Red resulted in some images such as the one in Figure 3.19 where the two markers were demarcated well. UPK was stained with DAB and PSA was stained with AMEC Red.

It is clear from figures 3.23 and 3.24, that even in well demarcated slides, it appears as though AMEC Red has stained with a lighter brown colour than DAB instead of the red colour. This presents a problem when it comes to interpretation as it is not crystal clear which is which. Furthermore, in some cases, such as the image of prostate glands shown in Figure 3.20 from another of the slides, the AMEC Red in fact stained a colour that was darker than even DAB staining – a comparison of which has also been shown with a stretch of urothelial tissue stained with UPK marked.

Such inconsistencies in staining patterns that occurred across the number of slides that were done made analysis or 3D reconstruction impossible simply because there were too many slides where the areas stained by AMEC Red and DAB were not discernible. One of the two – more likely the AMEC Red because validation had been done with DAB – also overstains the slides so validation with AMEC Red and the exact concentration to be used needs to be determined as it is not the same as that recommended by the manufacturer.

Just like the Opal kit, the work done with m-IHC also shows regions where both prostate epithelium and urothelium meet. However, the lack of clarity in each of the two stains being defined means that not a lot of images can be used successfully to interpret the data. However, for slides that have relative clarity – such as the one above, a better way to look at these images would be to use the “Replace Colour” feature on Photoshop and convert these into images that look like immunofluorescence images – again, drawing inspiration from SIMPLE.

One such image generated is shown in Figure 3.25 along with the image obtained from Aperio. Through these two images shown, it becomes clear that even though the two stains may appear similar, they are indeed different. In the IF-like image, DAB brown which is the colour that uroplakin is stained has been replaced by red and AMEC Red

has been replaced by green on a black background. However, despite this, it has to be noted that in areas such as that pointed out by the arrow on the second image Photoshop fails to recognise some shades of red as red and indeed thinks that it is brown – which leads to an outline of red on green. This is indeed the same problem that was discussed earlier with Photoshop. Moreover, it is impractical to do this on each and every slide before doing 3D reconstruction.

4.9. Summary of Findings

All in all, the studies done during this project beginning with optimisation of antibodies with IHC and ending with multiplex immunohistochemistry (m-IHC) enable us to draw a few conclusions.

1. Optimisation of various antibodies with IHC is helpful as most of the markers can be carried forward at the same concentration to the Opal kit without issue.
2. Heat plays a major role in determining which markers could be used for a multiplex image if using the Opal kit and this is sometimes a major stumbling block. As we have seen from the studies done by Lee et al., the effect of heat on the intensity of fluorescence cannot be ignored. Some of the markers as seen in this study decrease in intensity with every round of being subjected to microwave heat.
3. Photobleaching effect with the Opal kit is far more pronounced than expected and affects image capture massively.
4. The Opal kit method has some inherent problems in it when it comes to imaging as well as was seen when UPK-1b, PSA and 34BetaE12 were all stained together. This occurs due to the nature of the Opal kit fluorophores binding to the tyrosine residues in the vicinity of the protein of interest causing a potential overlap effect in the final image.
5. The Opal kit did however lend some positive results when only PSA and UPK were used. As the two markers were mutually exclusive, it was easy to see that they marked out prostate epithelium and urothelium respectively without overlap in some sections of tissue. However, the inconsistency in the results obtained with the Opal kit necessitated other methods to be considered
6. The positive results of the Opal kit most importantly showed regions of cells which did not take up either PSA or UPK. These regions could potentially be composed of stem cells but definitive proof will need staining with DLK-1. Moreover, there is also a need to correlate these findings with that of other

papers published in the field – most notably by Henry et al. (2019) and see whether any of their findings correlate with these.

7. With SIMPLE and DAB-based modification of SIMPLE, the problem arose with the use of Adobe Photoshop 2020 to combine different stages of imaging whereby both the “Replace Colour” and “Merge Image” functions had problems. However, one key finding was that it did confirm results from the Opal kit in some slides whereby there were regions where the urothelium and prostate epithelium overlapped and were in close proximity to each other.
8. Multiplex-IHC was the final method employed but a lack of contrast between the two substrates used compounded the problem when Adobe Photoshop was used whereby the software did not recognise some shades of red as being different from brown and vice versa. Converting the image to an IF-like image with a dark background helps only to a certain degree before running into problems.

The definitive conclusion we can take from these studies is that there is definitely some kind of close anatomic relationship between the prostate epithelium and the urothelium with there being cells present in areas between the two which are not positive for PSA or UPK. Now whether these are artefacts or legitimately stem cells can only be definitively identified by further staining with DLK-1 or other markers such as SCB1A1.

4.10. Strengths and Limitations of This Study

4.10.1 Strengths

1. The methodology of the study is in a sequential order whereby each antibody is validated with each method and then depending on the result of the optimisation and validation process, it is decided whether or not to carry on with it. Hence the sequential and progressive optimisation and elimination of markers leaves no room for error or misjudgment.
2. The problems of the Opal kit had become apparent with this method – something that was not evident from just a superficial look at the method. Various workarounds were tried but the inconsistency of the results meant that the Opal kit ultimately had to be abandoned as a viable method to determine the architecture of the prostate stem cell niche.

3. A similar issue arose with m-IHC where the two substrates used (DAB and AMEC-Red) theoretically were supposed to give good results but as it turned out, were not as effective.

The main strength of these studies thus translates to one which tells us of methods which will not be good for studying the prostate stem cell architecture. Through these studies, we have found out that the Opal kit has inherent issues such as epitope denaturation, hindrance and masking (findings which have also been seen in a study done by Lee et al., 2020) making it unsuitable to multiplex imaging with regard to the prostate.

While AMEC-Red and DAB do not form a good combination, I would say that the jury is still out on m-IHC because there are other substrates that can be tried out in place of AMEC-Red with the potential of positive results. Furthermore, m-IHC also offers a way for multiplexing more than just two markers eventually but the question of whether heat would affect the nature of epitopes would again be a question that would need to be addressed.

In terms of results, the main strength of these studies is in the fact some of the results from the Opal kit clearly show an overlap and some sort of interaction between the prostate epithelium and the urothelium. There are also some areas which seem to be neither urothelial nor prostate epithelial in nature. These cells neither take up PSA nor UPK meaning there could be a different type of cell that exists in that space – something that is in line with the findings of Henry et al. (2019) as well. While this study has not definitively proven the existence of stem cells in that region and the mapping out of that area over 3D reconstruction to see how far into the tissue these stem cell layers could go, it does increasingly point to the existence of such areas at the prostate epithelium-urothelium junction – in accordance with findings by Moad et al.

4.10.2. Weaknesses

One of the weaknesses of this study is the lack of conclusive findings in favour of or against the existence of stem cells. However this was mainly due to the fact that the methods used – the Opal kit, DAB-SIMPLE and m-IHC all had limitations of their own which have been discussed at length above. So rather than it being a problem with the hypothesis itself, I believe that this is more of an issue of the methodology and that it is only a matter of time before the hypothesis is either proven or disproven.

Another weakness of this study is that because of multiple factors such as problems associated with methodology and because COVID-19 lockdowns, the progress of the project has been affected. This means that I was unable to further study some other structural and functional aspects of the stem cell niche which I intended to do.

One other weakness of this project is the fact that the Opal kit was chosen ahead of m-IHC. If I could do the whole thing again, I would change that and do m-IHC first (and with different substrates) before moving on to the Opal kit because the procedure for m-IHC is basically the same as a standard DAB-IHC procedure except that it is repeated multiple times and with different substrates. Another reason this makes sense is because even though the Opal kit uses the same concentration of antibody after optimisation with IHC, it would be better if m-IHC followed IHC as it is essentially the same protocol. Plus because m-IHC relies on immunohistochemistry as opposed to immunofluorescence for visualisation of slides, the slides would be permanent and the fluorescence wearing off over time would not be an issue.

4.11. Future Work

4.11.1 Structural Studies

The next thing to do with the project is to further the use of m-IHC to determine the boundaries between the prostate epithelium and the urothelium. With AMEC-Red and DAB there are certain slides that did demonstrate potential boundaries. This could be exploited further by using different substrates that give more unrelated colours as brown and red being closely related was an issue. Instead, other substrates such as Vector Blue could be tried. This might give a good contrast between blue and brown in the right concentrations – making discerning boundaries easier.

There is a possibility of using Imaging Mass Cytometry (IMC) as well to look at images. The advantages with this is that the imaging is highly accurate. The disadvantage with this is that because it uses laser ablation as part of its mechanism – a slide can only be viewed once although the image may be stored forever. Moreover, at any given time, only a small area can be viewed with such an expensive procedure. Ideally, in the case of prostate stem cells and their niches, one would need to know their exact location prior to using IMC or else the procedure will be painstakingly long and expensive. Optimisation of antibodies would need to be done again for this using immunofluorescence as well.

Further then the areas which do not take up either PSA or UPK-Ib need to be looked at as potential stem cells or as potential cells of another nature that we may need to investigate further. This can be done by staining using m-IHC for markers of stem cells such as DLK-1. They also need to be investigated for their potential similarity to OE1 and OE2 as detailed by papers from Henry et al. (2018). This can also be done using antibodies against specific markers.

Following visualisation of the stem cells in each slide, they can then be put together to generate a 3D reconstruction image which will then help us deduce the architecture of the stem cell niche and just how it maps out the 3D environment physiologically.

4.12.2.Functional Studies

In addition to studies about the structure of the stem cell niche, work also needs to be done regarding the functional aspects of the niche.

One of the key findings from Moad et al. was that the prostate epithelium and urothelium form interdigitations amid which are located the stem cells and their niches. What would be a good exercise would be to conduct co-culture studies generating prostate epithelium and urothelium and see if there are certain pathways that can be exploited for therapeutic purposes

The role of certain pathways and markers in the prostate stem cell niche and potentially even in prostate cancer need to be investigated as well. Deiodinase-II is one that we could potentially look at as well with the added advantage that there already exists a tissue microarray in the lab that will help in studying the role of this particular thyroid marker in the prostate. Co-culture studies with prostate epithelium and urothelium or at least functional studies with regard to DiO2 or the Notch pathway-DLK relationship potentially involving prostate organoid studies and correlation with TMAs for thyroid hormones in the prostate could also have been done.

The presence of DLK-1 also raises an interesting question in terms of its role as a noncanonical Notch ligand. *In vivo,* DLK-1 suppresses the Notch pathway but its role *in vitro* needs to be investigated further. The exact nature of the interaction of the Notch pathway with respect to differentiation of stem cells and the multiplication of stem cells is something that can be looked at further.

Furthermore, there is also the question of cellular dynamics within the niche. With respect to prostate cancer, there is a predominantly peripheral presentation of the

disease. Now that could potentially mean two things with respect to stem cells. One, that the mutations needed for cancer are accumulated as the stem cells differentiate and migrate further. The second is that even if the mutations necessary for cancer are accumulated quite early on – possibly even within the stem cell niche, the microenvironment is not suitable to the formation of a tumour. Hence the tumour manifests itself only after the cells migrate to the periphery. The acquisition of the mutations itself is an interesting question and it could potentially be accumulated during cell division – especially if it involves asymmetric cell division. This is also something that could form an interesting study as well.

Chapter 5: References

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Chapter 6: Appendix

6.1. Publication: First Author - Human Prostate Stem Cells and Their Niche - A Comprehensive Review

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Abstract

Several recent major findings in the field of adult prostate stem cells have advanced our understanding of the cell biology. Earlier seminal studies in the murine prostate demonstrated and defined the cell biology and dynamics. However, it remained unclear how these findings correlated to the human prostate stem cell until very recently. The location and dynamics of the human adult prostate stem cell niche have now been identified. This has implications for further research into the origins of benign prostatic hypertrophy and prostate cancer. This review summarises the current evidence for prostate stem cells and their niche in both the murine and human models.

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Review Article

Human Prostate Stem Cells and Their Niche - A Comprehensive Review

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Abstract

Several recent major findings in the field of adult prostate stem cells have advanced our understanding of the cell biology. Earlier seminal studies in the murine prostate demonstrated and defined the cell biology and dynamics. However, it remained unclear how these findings correlated to the human prostate stem cell until very recently. The location and dynamics of the human adult prostate stem cell niche have now been identified. This has implications for further research into the origins of benign prostatic hypertrophy and prostate cancer. This review summarises the current evidence for prostate stem cells and their niche in both the murine and human models.

Keywords: Adult stem cell; Niche; Progenitor; Prostate; Stem cell

Introduction

The past decade has seen rapid developments in our understanding of adult stem cell biology and the central role they play in tissue maintenance, ageing and disease [1-3]. These insights are now providing new approaches to tissue regeneration, disease modelling and treatments [4].

In this review we focus on the prostate, where benign and malignant disease is common. Symptomatic Benign Prostatic Hypertrophy (BPH) affects more than 40% of men over 50 [5], whilst prostate cancer is the most common male cancer and the second leading cause of cancer death worldwide [6]. A better understanding of the normal mechanism of prostate epithelium maintenance will underpin new approaches to treatment as this will lay the foundations for understanding the effects of ageing and abnormal regulation in disease. The mouse prostate field has predominantly defined stem cells and mechanisms for homeostasis in pioneering techniques involving lineage tracing [7,8] and organoid culture [9,10]. How the findings from these studies correlate to human prostate stem cell biology remained an open question until very recently.

This review will focus on recent advances in describing human prostate stem cell biology, the prostate stem cell niche and the opportunities these findings afford for a translational impact.

Evidence for Human Adult Prostate Stem Cells - Basal, Luminal or Both?

The existence of somatic adult stem cells has been widely illustrated within various human organs, including hair follicles [11], skin [12], bone marrow [13] and intestinal tissue [3], which allow for tissue maintenance during ageing and rapid regeneration in cases of injury.

The initial evidence for stem cells within the prostate came from studies in rat, or murine, prostatic epithelium. The prostate epithelium consists of 3 types of cell: basal, luminal and neuroendocrine. The luminal cells line the ductal lumens, whilst the basal cells lie underneath, adjacent to the basal membrane [14]. Neuroendocrine cells are rare, secretory cells expressing neuropeptides and are distinct from the basal and luminal cells [14,15].

The first studies showed that androgen deprivation led to regression of adult prostate by preferential apoptosis of luminal cells. Re-administration of androgens then led to regeneration of new, fully developed prostate [16,17]. Another piece of evidence came, again from murine models. This time from transplantation of embryonic murine p63+ Urogenital Sinus (UGS) into immunodeficient mice [18]. The UGS then differentiated into luminal and neuroendocrine cells, but not basal, suggesting basal cells are not needed for generation of prostate-like tissue in transplantation assays. This study was followed closely by murine chimaeric studies between wild type and p63 knockout mice by Signoretti et al. [19]. They demonstrated that prostate epithelium, including basal and luminal cells, were derived only from p63+ mice, suggesting that during normal murine embryonic development all prostate cells arise from p63+ progenitors. Together, these studies suggest the presence of castrate-resistant adult prostate stem cells within the prostatic epithelium.

These studies ignited a search for the location of the stem cell. As p63 null mice do not develop a prostate, and p63 is expressed in basal cells only, it was suggested that both basal and luminal cells arise from basal p63 progenitors in the embryonic model [19]. By this logic, the search for an adult prostate stem cell was focussed on the basal compartment. Leong et al. demonstrated that a single adult basal cell can regenerate prostate tissue on transplantation, supporting the existence of adult basal multipotent prostate stem cells [20].

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At the same time, functional prostate regeneration assays demonstrated that a small population of basal cells in both human and murine prostate exhibit multipotency [20-24]. However, it is worth noting that these assays do not reflect the normal physiological condition.

Molyneux et al. demonstrated that determination of the cell of origin in the absence of functional lineage tracing studies would lead to misleading results [25]. Previous methods i.e transplantation of UGS [26] and in vitro methods of primary prostate culture [27], although both demonstrate bipotency of basal cells, they favour basal cell growth over luminal. This left the luminal cells relatively unexplored. Wang et al. demonstrated that a murine luminal stem cell existed, and was a cell of origin for prostate cancer [28]. However, this did not definitively demonstrate the existence of a luminal stem cell in the normal prostate as this was performed in a castrate resistant model, therefore not reflective of normal physiology.

The turn of the decade saw a revolution in technology with the emergence of lineage tracing studies, which have become the gold standard in stem cell research. The initial murine lineage tracing studies by Wang et al., demonstrated that Nkx3.1 is mainly expressed in luminal cells with expression in a small minority of basal cells. However, when the mice were castrated, they found that Nkx3.1 expression in luminal cells was much reduced [28,29]; these were termed castration-resistant Nkx3.1-expressing cells (CARNs). They then demonstrated that CARNs can generate both basal and luminal cells. However, this model did not represent the normal physiology, given the castration.

Two further, seminal lineage tracing studies in the murine model by Ousset et al. and Choi et al. were able to examine both luminal and basal cells for the potential of stemness, without predominance of basal cells. Choi et al. demonstrated by labelling cytokeratins 14 (CK14; basal-specific) and 8 (CK8; luminal-specific), in vivo, that neither lineage was able to differentiate into the other, suggesting the existence of independently sustained populations of unipotent luminal and basal stem cells in the adult murine model [30]. In the work by Ousset et al., they examined the embryonic and adult model, using the same labels as Choi et al. (CK14 and CK5 for basal cells and CK8 for luminal cells) [7]. They found in early developmentthat CK14 labelled basal cells demonstrated a large expansion and differentiation into both CK5+ basal and CK8+ luminal cells. CK5 lineage tracing demonstrated a far higher proportion of basal cells than luminal cells, whilst CK8 luminal cell lineage tracing demonstrated a stable frequency of luminal labelled cells but no basal differentiation. Lastly, they demonstrated a proportion (15%) of 'intermediate' adult cells. These are basal cells expressing both CK5 and CK8, which are strongly associated with luminal differentiation. Here, the existence of multipotent basal progenitors during prostate postnatal development contrasts with the distinct pools of unipotent basal and luminal stem cells that mediate adult prostate regeneration.

Together, these findings suggest the presence of unipotent luminal progenitors in both the embryonic and the adult mouse, whilst basal cells lose their embryonic multipotency and become predominantly unipotent in the adult. However, there appears to be a minor population amongst the luminal and basal cells that retain some potential for multipotency (Figure 1).

The study of human prostate stem cells was lagging behind that of the murine model, as lineage tracing models were limited until the discovery of the ability to use mitochondrial (mt) DNA mutations marks linked with ageing. These lead to respiratory chain deficits that can be measured by Cyclo-Oxygenase activity (CCO) [31,32]. The study by Ousset et al. in the murine model echoed the initial results in humanclonal analysisin prostatectomy specimens showing the presence of bipotent clones containing both basal and luminal CCO-deficient cells, as well as unipotent basal or luminal clones of CCO deficient cells [31,32].

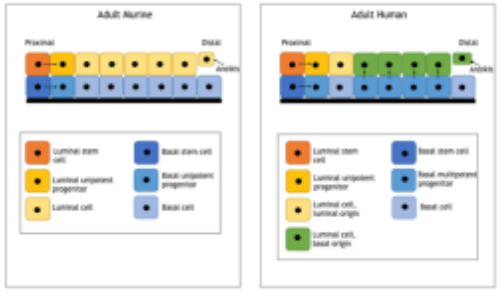

Figure 1: Diagram demonstrating adult murine and human prostatic epithelium.

A land mark paper came from Karthaus et al. [10], who argued that in vitro culture systems (UGS transplantation and primary prostate cell culture) don't generate tissues resembling the in vivo composition or contain Androgen Receptors (AR) at physiological levels. Utilising novel organoid culture methods pioneered in the investigation of the gastrointestinal tract [33,34], they produced sustainable R-spondin1 based adult prostate organoids from mouse and human prostate cells, respectively. The human model's luminal cells expressed physiological CK8 and AR, whilst basal cells expressed p63 and CK5, both in keeping with their epithelial position. The CK5-expressing basal and CK8-expressing luminal cells were then separated and cultured as organoids. Basal (CK5)-derived organoids expressed mostly CK5, with CK8 cells surrounding a sporadic array of lumens, with patchy AR expression. Luminal derived organoids immediately formed lumens. The majority of cells expressed CK8 and AR, with a small minority being CK5+, CK8- i.e basal cells. This indicates that human luminal cells are able to generate basal cells and vice versa i.e both luminal and basal stem cells have bipotency within the organoid model [10]. Finally, Moad et al. [35], using lineage tracing methods of human prostatectomy specimens with subsequent in vitro organoid culture, demonstrated multipotency of basal stem cells and unipotency of luminal stem cells (Table 1).

Model		Embryonic/Adolescent	Adult
Murine	*In Vitro*	Burger et al., 2005; Goldstein et al., 2010; Lawson et al., 2007; Leong et al., 2008; Xin et al., 2003 [20-24]	Leong et al. 2008; Karthaus et al. 2014 [10,20]
	In Vivo	Ousset et al. 2012 [7]	Wang et al. 2009; Ousset et al. 2012; Choi et al. 2012 [7,28,30]
Human	*In Vitro*	N/A	Karthaus et al. 2014; Moad et al. 2017 [10,35]
	In Vivo	N/A	Moad et al. 2017 [35]

Table 1: Summary of evidence for prostate stem cells in differing models.

We therefore have definitive evidence in humans for multipotency in basal stem cells and conflicting evidence of multipotency (organoid model) vs. unipotency (lineage tracing and organoid culture) in luminal stem cells.

Prostate Stem Cell Niche - Proximal or Distal?

As adult stem cells are required for the life of the tissue they are thought to reside in highly specialized, spatially confined locations, which provide unique microenvironments to maintain these essential cells; the stem cell niche. A functional niche should maintain a homeostatic balance between cellular quiescence and activity [2].

Until the work by Moad et al. [35], very little was understood about the true location, and definitive identity of human prostate stem cells and their niche. Prior to this, the stem cell niche had been described in other tissues, but not for the prostate. The relationship between other epithelial stem cells and their surrounding tissues (Extracellular Membrane [ECM], stromal cells, neighboring epithelial cells and possibly immune cells) had been described in differing tissues, notably the gut with intestinal crypts [2,36,37]. Intestinal crypts lie at the bottom of epithelial invaginations at the base of each villus within the small intestine [38]. This provides a physical space in which the cells can be contained and maintained.

The adult human prostate stem cell niche has only been described recently. As mentioned above, Moad et al. [35] demonstrated the multipotency of basal stem cells and unipotency of luminal stem cells using lineage tracing. Following from this, they performed 3D glandular reconstructions with proliferation kinetics and functional assays of differentiation. These demonstrated several key findings. Firstly, stem cells generated continuous migratory streams flowing from individual stem cells located in the basal layer of proximal ductal epithelium to peripheral distal epithelium. This finding of proximal stem cell location echoed the findings from animal studies, where Tsujimura et al. [39] demonstrated that murine prostate stem cells originate in the proximal portion of the duct and more recently a scRNA sequencing-based comprehensive cellular atlas of whole prostates revealed proximally residing putative progenitor cells (Club and Hillock cells). These proximal basal progenitors differentiate into both basal and luminal epithelial cells in all but the most proximal region [35]. Secondly, they made the observation that these basal progenitors never gave rise to proximally located luminal cells. These particular cells have their own, unipotent cells within the most proximal part of the duct, which give rise to short luminal-only clonal expansions. Thirdly, they demonstrated that the urothelium interdigitates with the prostatic epithelium into the proximal prostatic ducts. The proximal basal progenitors lie at the interface between the urothelium and prostatic epithelium within these separating interdigitations, topographically-similar in structure to intestinal crypts.

Together these findings show that human prostatic stem cells originate in the basal layer in the stem cell niche nested within interdigitationsof urothelium and flow, unidirectionally into the distal ducts, where they differentiate into both luminal and basal cells. A separate, limited pool of luminal stem cells maintains the luminal epithelium at the most proximal part of the duct (Figure 1).

Stem Cell Dynamics in the Prostate Stem Cell Niche

Stem cell dynamics, or modes of cellular division, is a key process in maintaining the stem cell population and providing differentiated cells for replenishment of specific tissues. Stem cell division can be either symmetrical, whereby two daughters cells of equivalent fates are produced, or asymmetrical where the daughter cells have differing fates, namely a stem cell and a committed progenitor of fully differentiated tissue.

Cell division within a closed niche, such as that demonstrated in the prostate [35] poses a problem, namely that there is spatial limitation, allowing only a finite number of stem cells to reside there. This phenomenon has been extensively investigated in the intestinal crypts. A seminal paper by Ritsma et al. [36], demonstrated that, contrary to previous studies, intestinal stem cell division appears symmetric. However, increasing loss of contact with the niche seems to prevent the maintenance of stemness and they begin to differentiate [36].

There is no such definite work on the prostatic niche, however it has been demonstrated in the murine prostate, by labelling centrosomes with γ-tubulin, that mitotic spindles among dividing basal cells occurred in two predominant orientations to the basement membrane: horizontal and vertical [40]. They observed that formation of the luminal layer was marked with an increased number of vertical divisions, with vertical division increasing from around 36% at postnatal day 5 to around 65% at postnatal day 15. Examination of horizontally and vertically oriented daughter cells revealed two distinct fates for the dividing basal cells (asymmetrical and symmetrical). Horizontally-divided basal cells always gave rise to two daughter basal cells (as detected by p63 staining), whilst vertically divided cells gave rise to a basal cell (p63 positive) and an apical, luminal cell (p63 negative, but CK8 positive). Luminal cells only gave rise to other luminal cells, by dividing horizontally. These findings fitted with previous observations of a multipotent basal progenitor and a unipotent luminal progenitor in the embryonic murine model [7].

Regulatory Pathways in the Prostate Stem Cell Niche

As described earlier, a stem cell niche not only provides an anatomical location, in this case in the prostatic epithelium surrounded by interdigitating urothelium at the proximal portion of the prostatic duct, but also a microenvironment, which supports and sustains the stem cell. Loss of contact to which, appears to induce loss of stemness [36], at least in the intestinal stem cell niche. A seminal study in mammalian skin first identified the importance of a Notch-dependent pathway in apparent asymmetric division in epidermal stem cells [12].

In a series of embryonic murine studies, Notch1 was initially identified in basal progenitor cells [41]. Notch1 was then shown to be indispensable for prostatic morphogenesis and regrowth following androgen deprivation and replacement [42]. Finally, [43] demonstrated that, by stimulating the Notch pathway directly, there was a stimulatory effect on cell differentiation and proliferation. These series of experiments demonstrated that the Notch pathway was a major regulator of embryonic murine prostatic basal stem cells. A further study in adult murine prostates, then demonstrated that Notch ligands were present in both the basal and luminal layers [44], consistent with contemporaneous studies showing separate pools of unipotent progenitors within the separate layers [7]. Disruption of the pathway increased differentiation and proliferation of the basal layer, but not the

luminal. Whilst application of ectopic Notch resulted in a decrease in basal cell number and luminal cell hyper proliferation [44].

As before, human research took time to catch up with that of the murine. Ceder et al. [45], demonstrated that DLK1, a 'dead' ligand [46] to Notch and known regulator of prostatic epithelial homeostasis [43,44], was highly expressed in adult human prostate basal stem cells. They found that these basal stem cells displayed little to no Notch reactivity [5]. A further study demonstrated that, like the mouse, Notch is an established regulator of basal progenitor differentiation [47].

It was not until the study by Moad et al. [35], that the evidence for the effect of Notch and DLK1 in adult human prostatic stem cells was clarified. With immunohistochemistry of DLK1 and Notch in vivo they demonstrated a distinct spatial expression profile, with DLK1 only expressed in basal cells within, the now identified, prostatic stem cell niche. This was consistent with DLK1 providing an inhibitory signal, opposing Notch-stimulated basal progenitor differentiation. DLK1 was also identified in peripheral, or distal, luminal cells, where the greatest apoptotic activity was identified, suggesting a gradient in Notch expression. This was consistent with previous findings showing DLK1 inhibits Notch-regulated resistance to cell death through anoikis (detachment from the extracellular membrane) [48]. Moad et al. [35] further tested this observation with experimental organoid culture of DLK1+ and DLK1- basal cells. DLK1+ basal-cell derived organoids developed into long-lasting mature prostatic architecture expressing basal (CK5) and luminal (CK8, PSA and AR) markers. Whilst, DLK1- organoids did not mature and there was visible apoptosis.

Another signalling pathway associated with prostatic stem cells is the Wnt pathway. Hu et al. [49] demonstrated, *in vitro*, that prostatic stem cells expressed high levels of Wnt10B, known to be a regulator of stem cell homeostasis [50]. As yet, this has not been demonstrated *in vivo*, but remains a tantalising research target.

These studies provide the latest evidence for how the adult human prostate epithelium is organized. Proximal basal stem cells propagate, differentiating distally into both basal and luminal cells, replacing distal luminal cells lost through apoptosis. It appears reasonable to hypothesize that the interaction between Notch and DLK1 induces a quiescent state in basal prostate stem cells, whilst loss of DLK1 distally and therefore loss of this interaction then drives luminal differentiation as shown in the murine studies [44]. DLK1 expression at the periphery, associated with apoptosis, suggests a Notch gradient, maximal at the niche, tapering to negligible in the periphery, although this, along with a role for the Wnt pathway have yet to be demonstrated.

Summary

In this review, we define the current understanding of the biology of the human prostate stem cell niche. Recent findings open new lines of investigation and we outline potential cell signaling regulators and cell-to-cell interactions of interest, which could be explored as potentially aberrant in diseased states. Furthermore, there is unique topography to the prostate niche which may provide increasing gradients to stem cell niche factors toward the apexes of the niche between the interdigitating urothelial tongues and will be the focus of future research investigating the role of the niche in prostate cancer.

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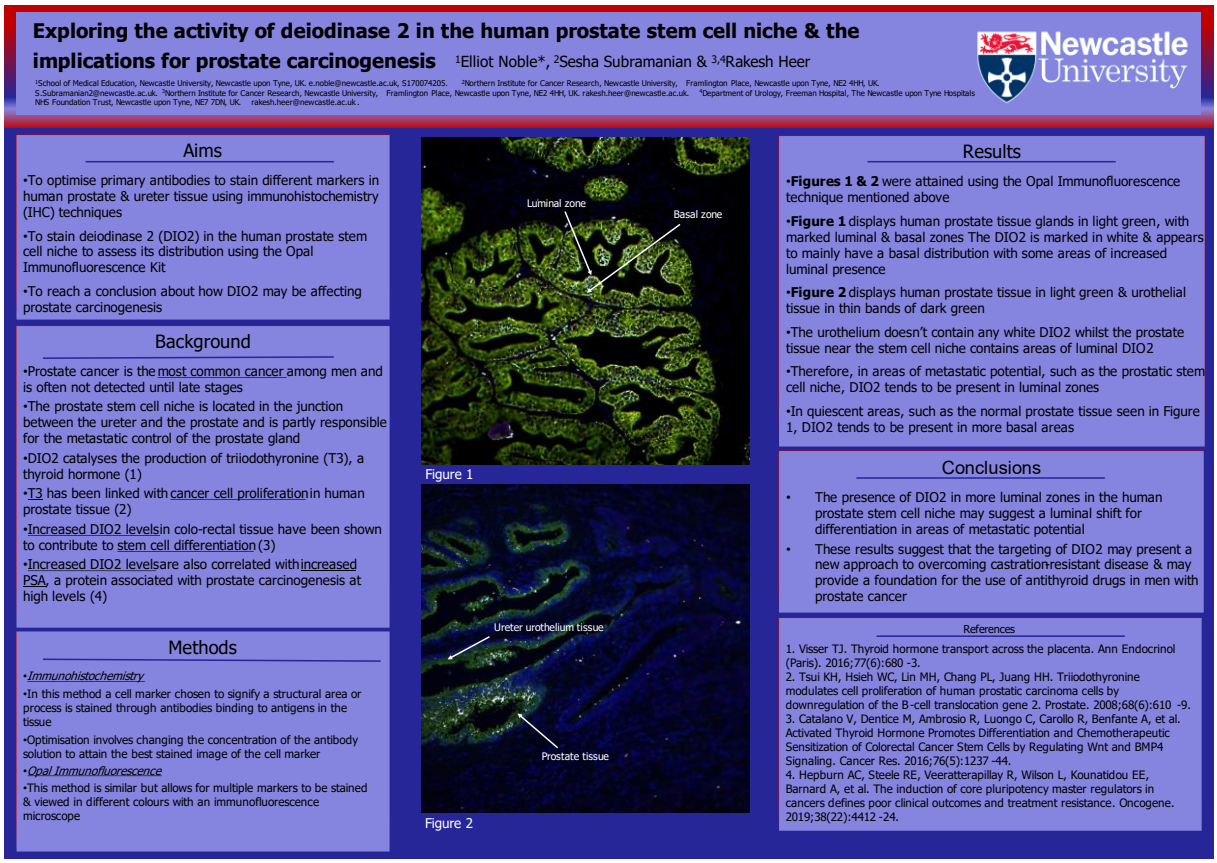
6.2. Poster: Secondary Author - Exploring the activity of deiodinase-2 in the human prostate stem cell niche and the implications for prostate carcinogenesis



6.3. Poster: Secondary Author - Identification of the Prostate Stem Cell Niche and Its Role in Carcinogenesis

Identification of Prostate Stem Cell Niche and its role in carcinogenesis

Zhen Xin Wong | Z.X.Wong3@Newcastle.edu.my | MBBS Stage 2 | 160657850
Supervised by : Dr Desha Subramanian and Prof Rakesh Heer

Newcastle University
Northern Institute for Cancer Research

Background

The **prostate gland** is an important male reproductive organ which is responsible for the production of semen. A **stem cell niche** is a microenvironment surrounding stem cells which regulates their activity to maintain the normal cell cycle. Hence, dysregulation of the stem cell niche can lead to unstoppable division which contributes to the formation of cancer.
Kuffel like factor-4 (KLF4) is protein that is highly expressed in non-dividing cells and its overexpression induces cell cycle arrest. KLF4 is important in preventing cell division when DNA is damaged which may be used as marker to predict cancer.

Microscopically, prostate gland consist of **basal cells, luminal cells** and **neuroendocrine cells** as shown in image 1. It has been proposed that **stem cells** are **more prevalent in basal cell layers** which can give rise to new basal and luminal cells. Macroscopically **stem cell niche is** hypothesised to be **located in the proximal location** of prostate gland instead of distal.

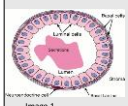


Image 1

Image 2

Aim

1. To identify the location of prostate stem cell niche
2. Investigate the relationship between KLF4 and prostate stem cell niche

Method

Immunohistochemistry (IHC) Optimisation
Slides containing prostate gland will undergo four processes which are **dehydration, blocking of nonspecific site** to prevent unnecessary binding **addition of primary antibodies** and lastly **dehydration and mounting** All the process are the same except the addition of primary antibodies as different primary antibodies stain for different parts in different cells. Slides are then viewed under the microscope.

Marker	Optimal Concentration *	Stained area
34 BetaE12	1:100	**Cytoplasm** of **basal** cell
PSA	1:5000	**Cytoplasm** of **luminal** cell
KLF	1:50000	**Nucleus** of **luminal** cell
Uroplakin	1:20	**Urothelium**
p63	1:100	**Nucleus** of **basal** cell
Androgen receptor	1:500	**Nucleus** of **luminal** cell
Ki67	1:100	Proliferative cell

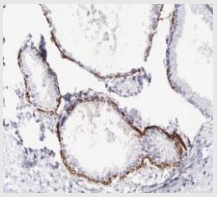
Chart 1 showing optimal concentration for each marker and the area that the marker mainly stained.
For optimal concentration, 1:100 means 1µL of primary antibody dissolved in 100µL of solvent

Optimal concentration for all the markers are developed through serial dilution for the primary antibodies. F example, for the marker PSA, different concentrations for primary antibodies are used (1:2000, 1:3000, 1:500 1:7000) and the optimal concentration is determined based on the images under the microscope.
The four highlighted marker are then repeated on prostate tissues using another method known **Opal method**. It allows for the staining of tissues using multiple primary antibodies (which cannot be performed o IHC procedure as one marker is allow on one slide in IHC procedure).

References
1. https://www.researchgate.net/figure/Schematic-illustrationof-the-anatomy-of-the-human-prostate-a-and-mouse-prostate-b_fig2_51824541
2. http://genesdev.cshlp.org/

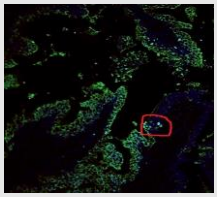
Result

IHC optimization



34Beta312 markers are used with optimal concentration of **1:100**. The cytoplasm of basal cells are stained clearly .

Opal kit method



The image above show prostate tissue undergone Opal method with few markers—PSA shown as bright green, uroplakin shown as red, KLF shown as aqua blue. The circle area is the junction between prostate and ureter which is the proposed area for stem cell niche.

Discussion

1. The Boundary between urothelium and prostate gland are identified where stem cell niche is proposed to be present. This is confirmed with the Ki67 marker (table 1) which stains proliferative cells and the circle area is shown to contain the most Ki67 marker compared to other areas. (image is not shown for this).

2. The aqua blue area shown in the circle (stem cell niche) indicates the reaction between cells with the KLF marker. Since the KLF4 marker is important in preventing cell division when the DNA is damaged, its regulation is important in preventing carcinogenesis. The relationship between the KLF marker and stem cell niche of prostate cancer warrants further study.

Result

Stem cell niche is present at the boundary of urothelium and prostate gland where highly proliferative stem cells are shown to be present **KLF marker** is shown to be **present in the stem cell niche** and is perhaps **important in regulating the normal stem cell niche**

Acknowledgement

Special thanks to **Dr Sesha** and **Prof Rakesh** for the helps throughout the research and also Newcastle University Northern Institute of Cancer for providing this opportunity. Gratitude are expressed to Vacation scholarship team for providing this scholarship too.

6.4. Ethical Approval for the Project

Newcastle Biobank

The Newcastle Upon Tyne Hospitals NHS
NHS Foundation Trust

Newcastle University

Newcastle University
Medical School
Framlington Place
Newcastle upon Tyne NE2 4HH

Date 06 March 18

Dear Rakesh

RE: Newcastle Biobank application NAHPB-138

Thank you for your application to the Newcastle Biobank for review. I am writing to inform you that your application was successful.

However, one important caveat applies to this approval, **you must not release any of the results obtained from these studies to the patients or to the clinicians treating them**. If you find a result which could have clinical implications please contact me to discuss the best course of action. You may feedback general information on whether the aims of the study could be achieved with any particular sample i.e. whether the sample was suitable etc.

Please contact Siaw Yein Ng, Bioresource Coordinator, (siaw.ng@ncl.ac.uk) for further information.

Your project may now run for 364 days. Applications may be submitted to re-register your project at yearly intervals; where annual fee applies. Re-registration takes into account the annual progress of the project. In view of this, we would like to take this opportunity to remind you that the Newcastle Biobank expects a yearly progress report on your study. Failure to submit this may jeopardise any further release of samples for your project if you do not provide a timely annual report. A copy of any publications should be sent to the Bioresource Coordinator and the Biobank should be acknowledged in the publication, as described in the Bioresource Access Policy.

Finally, you should keep any material that is surplus to your research study in trust on behalf of the Biobank for other approved studies; the Bioresource Coordinator must be informed if you hold any such material at the end of a study.

Best wishes and good luck with your studies,

Yours sincerely,
Dr Siaw Yein Ng on behalf of Dr Chris Morris

The Queen's Anniversary Prizes
2009

CONDITIONS OF ETHICAL APPROVAL

Research Ethics Committee:	North East - Newcastle & North Tyneside 1 Research Ethics Committee
Research Tissue Bank:	Newcastle Biobank
REC reference number:	17/NE/0361
Name of applicant:	Dr Christopher M Morris
Date of approval:	9 February 2018
IRAS project ID:	233551

Ethical approval is given to the Research Tissue Bank ("the Bank") by the Research Ethics Committee ("the Committee") subject to the following conditions.

1. Further communications with the Committee

1.1 Further communications with the Committee are the personal responsibility of the applicant.

2. Duration of approval

2.1 Approval is given for a period of 5 years, which may be renewed on consideration of a new application by the Committee, taking account of developments in legislation, policy and guidance in the interim. New applications should include relevant changes of policy or practice made by the Bank since the original approval together with any proposed new developments.

3. Licensing

3.1 A copy of the Licence from the Human Tissue Authority (HTA) should be provided when available (if not already submitted).

3.2 The Committee should be notified if the Authority renews the licence, varies the licensing conditions or revokes the Licence, or of any change of Designated Individual. If the Licence is revoked, ethical approval would be terminated.

4. Generic ethical approval for projects receiving tissue

4.1 Samples of human tissue or other biological material may be supplied and used in research projects to be conducted in accordance with the following conditions.

4.1.1 The research project should be within the fields of medical or biomedical research described in the approved application form.

4.1.2 The Bank should be satisfied that the research has been subject to scientific critique, is appropriately designed in relation to its objectives and (with the exception of student research below doctoral level) is likely to add something useful to existing knowledge.

4.1.3 Where tissue samples have been donated with informed consent for use in future research ("broad consent"), the Bank should be satisfied that the use of the samples complies with the terms of the donor consent.

4.1.4 All samples and any associated clinical information must be non-identifiable to the researcher at the point of release (i.e. anonymised or linked anonymised).

4.1.5 Samples will not be released to any project requiring further data or tissue from donors or involving any other research procedures. Any contact with donors must be confined to ethically approved arrangements for the feedback of clinically significant information.

4.1.6 A supply agreement must be in place with the researcher to ensure storage, use and disposal of the samples in accordance with the HTA Codes of Practice, the terms of the ethical approval and any other conditions required by the Bank.

4.2 A research project in the UK using tissue provided by a Bank in accordance with these conditions will be considered to have ethical approval from the Committee under the terms of this approval. In England, Wales and Northern Ireland this means that the researcher will not require a licence from the Human Tissue Authority for storage of the tissue for use in relation to this project.

4.3 The Bank may require any researcher to seek specific ethical approval for their project. Such applications should normally be made to the Committee and booked via the Central Booking System

4.4 A Notice of Substantial Amendment should be submitted to seek the Committee's agreement to change the conditions of generic approval.

5. Records

5.1 The Bank should maintain a record of all research projects to which tissue has been supplied. The record should contain at least the full title of the project, a summary of its purpose, the name of the Chief Investigator, the sponsor, the location of the research, the date on which the project was approved by the Bank, details of the tissue released and any relevant reference numbers.

5.2 The Committee may request access to these records at any time.

6. Annual reports

6.1 An annual report should be provided to the Committee listing all projects for which tissue has been released in the previous year. The list should give the full title of each project, the name of the Chief Investigator, the sponsor, the location of the research and the date of approval by the Bank. The report is due on the anniversary of the date on which ethical approval for the Bank was given.

6.2 The Committee may request additional reports on the management of the Bank at any time.

7. Substantial amendments

7.1 Substantial amendments should be notified to the Committee and ethical approval sought before implementing the amendment. A substantial amendment generally means any significant change to the arrangements for the management of the Bank as described in the application to the Committee and supporting documentation.

7.2 A Notice of Substantial Amendment should be generated by accessing the original application form on the Integrated Research Application System (IRAS).

7.3 The following changes should always be notified as substantial amendments:

7.3.1 Any significant change to the policy for use of the tissue in research, including changes to the types of research to be undertaken or supported by the Bank.

7.3.2 Any significant change to the types of biological material to be collected and stored, or the circumstances of collection.

7.3.3 Any significant change to informed consent arrangements, including new/modified information sheets and consent forms.

7.3.4 A change to the conditions of generic approval

7.3.5 Any other significant change to the governance of the RTB.

8. Serious Adverse Events

8.1 The Committee should be notified as soon as possible of any serious adverse event or reaction, any serious breach of security or confidentiality, or any other incident that could undermine public confidence in the ethical management of the tissue. The criteria for notifying the Committee will be the same as those for notifying the

Human Tissue Authority in the case of research tissue banks in England, Wales and Northern Ireland.

9. Other information to be notified

9.1 The Committee should be notified of any change in the contact details for the applicant or where the applicant hands over responsibility for communication with the Committee to another person at the establishment.

10. Closure of the Bank

10.1 Any plans to close the Bank should be notified to the Committee as early as possible and at least two months before closure. The Committee should be informed what arrangements are to be made for disposal of the tissue or transfer to another research tissuebank.

10.2 Where tissue is transferred to another research tissue bank, the ethical approval for the Bank is not transferable. Where the second bank is ethically approved, it should notify the responsible Research Ethics Committee. The terms of its own ethical approval would apply to any tissue it receives.

11. Breaches of approval conditions

11.1 The Committee should be notified as soon as possible of any breach of these approval conditions.

11.2 Where serious breaches occur, the Committee may review its ethical approval and may, exceptionally, suspend or terminate the approval.