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Getting down to specifics: profiling gene expression and protein-DNA interactions in a cell type-specific manner

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Abstract

The majority of multicellular organisms are comprised of an extraordinary range of cell types, with different properties and gene expression profiles. Understanding what makes each cell type unique, and how their individual characteristics are attributed, are key questions for both developmental and neurobiologists alike. The brain is an excellent example of the cellular diversity expressed in the majority of eukaryotes. The mouse brain comprises of approximately 75 million neurons varying in morphology, electrophysiology, and preferences for synaptic partners. A powerful process in beginning to pick apart the mechanisms that specify individual characteristics of the cell, as well as their fate, is to profile gene expression patterns, chromatin states, and transcriptional networks in a cell type-specific manner, i.e. only profiling the cells of interest in a particular tissue. Depending on the organism, the questions being investigated, and the material available, certain cell type-specific profiling methods are more suitable than others. This chapter reviews the approaches presently available for selecting and isolating specific cell types and evaluates their key features.

Keywords

Cell type-specific profiling; cell isolation; transcriptomics; model organisms; ChIP; Targeted DamID (TaDa); FACS; MACS; BiTS-ChIP; INTACT

1. Introduction

The generation of cells with different properties is a crucial step during the development of a multicellular organism. In embryogenesis, differentiation initiates as early as the 2-cell stage in mice (Biase, Cao & Zhong, 2014), whilst in *Drosophila*, germ cells are specified as rapidly as within 1.5 hours post-fertilisation (Sonnenblick, 1950). As embryogenesis and adult development continue, a greater diversity of cells are produced. While inter-tissue variation is particularly obvious, a great deal of diversity is observed even within individual tissues, for example the central nervous system (CNS). Examining the transcriptional and epigenetic profiles of specific cells during development can provide key insights into the gene regulatory mechanisms that specify individual cell types, how these cells react to developmental signals, and their mitotic potential over time. Correspondingly, it would be

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very challenging to obtain a comprehensive overview of how an embryo or tissue is formed and characterised without this detailed knowledge.

Understanding cell type-specific processes is not just important for the field of developmental biology, but can provide insights into the investigation of adult physiology (both healthy and diseased) and the oncogenic potential of different cell types within tumours. Tumours are often comprised of a very heterogeneous population of cells, with varying potentials to divide, of evading the immune system, and in promoting angiogenesis (Meacham & Morrison, 2013). Pinpointing and targeting specific cancer cells based on their molecular profile has, and will continue to, assist(ed) researchers to develop customised strategies for combating individual cancer types. For example, cancer stem cells are now being targeted within tumours, with the aim to reduce the chance of relapse (Chen, Huang & Chen, 2013).

Large-scale expression profiling screens have given us an insight into the variety of gene expression in model organisms, both spatially and temporally. The modENCODE consortiums have profiled global mRNA levels and epigenetic marks in whole *C. elegans* and *Drosophila* organisms throughout their development (Gerstein et al., 2010; Graveley et al., 2011). In addition, whole tissues have been profiled for *Drosophila* (Chintapalli, Wang & Dow, 2007; Graveley et al., 2011; Nègre et al., 2011; Chintapalli et al., 2012). These studies have provided some key insights into the developmental timing of gene expression and chromatin states, as well as tissue specific profiles producing very useful references for *Drosophila* researchers. However, especially with whole organism studies, a substantial amount of detail and context is unavailable since signals are averaged across many different cell types. Alternative resources for investigating expression patterns are the high-throughput RNA *in situ* projects. These include the *Drosophila* embryo BDGP expression pattern database (Tomancak et al., 2002) and the Allen brain atlas (Lein et al., 2007). The Allen Institute for Brain Science (<http://www.brain-map.org/>) is examining mRNA expression patterns in mouse, rodent and human nervous system tissues as well as in embryos. These are powerful resources for the research community; however, they also have their limitations; often not providing single cell resolution, assessing only mRNA expression, and the data consisting of a more qualitative than quantitative format.

Given the recent and continuing progress in the fields of genomics and developmental biology, more researchers are asking what is happening at the genomic level within individual cell types in a specific organism or tissue. For example: What mRNA is being expressed? What mRNA is being translated? What is the histone code profile? And what is the topology of the chromatin packaged into the nucleus? To answer these, and more hypothesis driven questions, a variety of approaches have been developed over the years (see Figure 1). These fall into two main categories; techniques which require cell/nuclei isolation and ones that do not. This chapter will review these methods and provide examples of how they have furthered our understanding of developmental biology, physiology and cancer.

2. Expressing transgenes for the purpose of cell type-specific profiling

The vast majority of methods used for cell type-specific profiling require the expression of some sort of transgene in the cells of interest. This is necessary for either sorting/isolating the cells, or to label/pull-down the RNA or DNA from the targeted subpopulation.

Transgenes can be expressed through a direct fusion of a promoter to the transgene-coding sequence, or by using a binary system, whereby the promoter is fused to a trans-acting factor, which in turn activates the expression of the effector transgene. In this section we provide an overview of the targeted expression approaches available for each of the common model systems.

2.1 *Drosophila* GAL4, LexA and QF expression systems

The GAL4/UAS binary system (Brand & Perrimon, 1993) is the most commonly used method for targeted gene expression in *Drosophila* (for reviews, see (Southall, Elliott & Brand, 2008; del Valle Rodríguez, Didiano & Desplan, 2012)). A wealth of GAL4 ‘driver’ lines, expressing the yeast transcription factor GAL4 in specific cell types, is now available. These ‘driver’ lines can be crossed to specific ‘responder’ lines, which possess upstream activator sequences (UAS) upstream of the transgene to be expressed. In the resulting *Drosophila* progeny, the transgene is expressed only in the cells where GAL4 is present (see Figure 2A). Due to the silence of the transgene in the absence of GAL4, responder lines can be generated without the complication of the phenotypic consequences due to misexpression, such as lethality. Additionally, the spatial activity of GAL4 in the organism can be further refined by the use of the GAL4 repressor, GAL80 (Ma & Ptashne, 1987; Lee & Luo, 2001). Employing the temperature sensitive version of the same protein, GAL80^{ts}, enables the temporal selectivity of expression (Matsumoto, Toh-e & Oshima, 1978; McGuire, Le, Osborn, Matsumoto & Davis, 2003) as well as through the drug inducible GeneSwitch system (Osterwalder, Yoon, White & Keshishian, 2001). Split-GAL4 can also be utilised to produce a more refined expression pattern of the effector, through the intersection of two promoters/enhancers (Luan, Peabody, Vinson & White, 2006).

A recent addition to the *Drosophila* tool kit is the Q system, using components identified from the fungus *Neurospora crassa* (Potter, Tasic, Russler, Liang & Luo, 2010). The Q system is comprised of the transcriptional activator QF, the QF effector QUAS, the QF suppressor QS, and the non-toxic drug quinic acid, which inhibits QS. The Q system can be temporally controlled, when QS is also expressed in the background, through the addition of quinic acid to the food. Recent modifications of the system have produced less toxic versions of QF (Riabinina et al., 2015). LexA-LexAop from λ phage is a third binary expression system (Lai & Lee, 2006) which has also been recently updated to utilise the QF activator domain for enhanced expression levels (Riabinina et al., 2015).

2.2 Mouse Cre/Lox, TetR and GAL4/UAS systems

The most common method to drive the expression of a transgene in a cell-specific manner, within mice is the Cre/lox system (Gu, Zou & Rajewsky, 1993). There are now a wide variety of transgenic mouse lines that express the site-specific DNA recombinase Cre in specific cell types (Heffner et al., 2012). Like the GAL4/UAS system, there is a driver line

(the mouse line expressing Cre in specific cells) and a responder line (a mouse line that will only express a transgene when Cre is present). However, the method by which the Cre/lox system works is different to GAL4/UAS, in that Cre, which is a DNA recombinase, facilitates the removal of a stop-cassette positioned between a ubiquitous promoter and the coding sequence of the transgene (see Figure 2B). A stop-cassette which comprises a neomycin cassette is often used (Soriano, 1999). When a Cre-driver line is crossed to a responder line, Cre will bind the *loxP* sites flanking the stop-cassette and excise it, allowing the ubiquitous promoter to drive expression of the transgene of choice in a permanent and heritable manner. In cells where Cre is absent, the stop-cassette is not excised and the promoter is unable to drive expression of the transgene. The Cre/lox system has now been adopted in the vast majority of eukaryotic model organisms due to its success as a site-specific recombinase (Vergunst, Jansen, Fransz, de Jong & Hooykaas, 2000; Lin, Lee, Wu, Duann & Chen, 2013; Hubbard, 2014). This form of genetic modification through recombination is very similar to the FLP/FRT system developed in *Drosophila* by Golic and Lindquist, which has now also been implemented in mice (Golic & Lindquist, 1989; Branda & Dymecki, 2004). For FLP/FRT, the recombinase Flippase (FLP) catalyses recombination between FLP recognition targets (FRTs) in a set-up similar to that described for Cre/lox to induce spatial, and even temporal, transgene expression and knock-outs (Hubbard, 2014).

Choosing an appropriate promoter is an important consideration when designing a responder line. The CAG (chicken beta-actin promoter and cytomegalovirus enhancer) (Niwa, Yamamura & Miyazaki, 1991) provides strong expression, especially in neural and heart tissues (Toyoda et al., 2003). Drawbacks of this promoter however, include a non-uniformity of expression across tissues (Griswold, Sajja, Jang & Behringer, 2011) and the fact that it can be silenced *in vivo* (Rhee et al., 2006). Other widely used ubiquitous promoters are the ROSA26 and UBC promoters (Schorpp et al., 1996; Kisseberth, Brettingen, Lohse & Sandgren, 1999). Homologous recombination can be used to knock-in the transgene directly into the ROSA26 locus and is the preferred option for uniform, ubiquitous expression within the embryo (Soriano, 1999). More recently, the CAG promoter has been incorporated into the transgene being inserted at the ROSA26 locus (Madisen et al., 2010; Snippert et al., 2010). This has been shown to boost expression levels, especially in adult tissues where ROSA26-driven expression is weak (e.g. the brain (Madisen et al., 2010)).

There are also transcriptional transactivation systems for mice (for a review see (Lewandoski, 2001)). The tetracycline-responsive system utilises a tissue-specific expressed TetR-VP16, which only activates transgene expression in the presence of the drug doxycycline (Gossen et al., 1995). This can be combined with Cre/lox (doxycycline-inducible Cre expression), so that the recombination, and subsequent expression, of a transgene (downstream of a *loxP*-flanked stop cassette) can be controlled temporally (Guo et al., 2005; Rao & Monks, 2009). Likewise, Imayoshi and colleagues developed a tamoxifen-inducible version of Cre/lox, Nes-CreER^{T2}, which, when crossed with mice harbouring ROSA26, enables relative temporal control in the developing nervous system (Imayoshi, Ohtsuka, Metzger, Chambon & Kageyama, 2006). Use of site-specific recombinase systems however, do enable the reversible activation or suppression of a transgene, as once recombination

event is complete, it cannot be undone. The GAL4/UAS system has also been employed for mouse studies (Ornitz, Moreadith & Leder, 1991; Echelard et al., 1993) and can be temporally regulated by the antiprogesterin RU486 (Wang, DeMayo, Tsai & O'Malley, 1997).

2.3 Zebrafish GAL4/UAS expression system

The GAL4/UAS expression system was adapted for use in zebrafish six years after its development in *Drosophila* (Brand & Perrimon, 1993; Scheer & Campos-Ortega, 1999). Since then, the system has been optimised by Distel and colleagues (Distel, Wullimann & Köster, 2009). Alterations to the GAL4 driver included the addition of a kozak sequence, modifications to the codon usage, and insertion of a rabbit β -globin intron to produce the modified GAL4, KalTA4GI. In addition, they optimised the number of UAS sites (x5) and demonstrated that it could be used for permanent labelling of specific cell types through an effector feedback loop.

Temporal control has been recently added to the zebrafish GAL4 toolkit with the development of an inducible system (Ramezani, Laux, Bravo, Tada & Feng, 2015). Similar to GeneSwitch in *Drosophila* (Osterwalder et al., 2001), KalTA4 is fused to a mutated ligand-binding domain from the human estrogen receptor, allowing its activity to be controlled temporally by the addition of the hormone 4-hydroxytamoxifen.

In the advent of CRISPR technology (Hsu, Lander & Zhang, 2014; Sander & Joung, 2014), zebrafish researchers are now using this versatile genome-editing tool to switch existing promoter-driven GFP transgenic lines to cell type-specific GAL4 lines (Auer, Duroure, Concordet & Del Bene, 2014; Kimura, Hisano, Kawahara & Higashijima, 2014). Furthermore, now that the tools and resources for GAL4/UAS driven expression in zebrafish are maturing, it is likely that more cell type-specific profiling technologies will soon be adapted for this model organism.

2.4 Cell type-specific expression of transgenes in *C. elegans*

In addition to *Drosophila*, another major invertebrate, metazoan model for genetic and physiological investigation is the free-living nematode, *Caenorhabditis elegans*. Traditionally, transgenic *C. elegans* have been generated through the formation of extrachromosomal arrays and the rescue of the *Dpy-5* mutant phenotype (Mello, Kramer, Stinchcomb & Ambros, 1991). The transgene is expressed from a cloned promoter and will be present in multiple copies in each cell. This is because the extrachromosomal array has to be of a sufficient size for it to be heritable (Stinchcomb, Shaw, Carr & Hirsh, 1985; Mello et al., 1991; Mello & Fire, 1995). With this method, the number of transgene copies can vary substantially with multiple copies occasionally resulting in prohibitively high levels of expression.

The *C. elegans* field is advancing from extrachromosomal arrays to alternative methods of introducing single copy insertions/deletions (eg. MosSCI (Frøkjær-Jensen et al., 2008)) or towards modifying endogenous genes using CRISPR-triggered homologous recombination (Dickinson, Ward, Reiner & Goldstein, 2013). However, binary expression systems have remained relatively undeveloped in *C. elegans*, as researchers have tended to use single

tissue-specific promoters for spatial control (McKay, McKay, Avery & Graff, 2003). Nevertheless, there is now a selection of binary expression systems available, including a FLP-Out system (Voutev & Hubbard, 2008), a heat-shock induced binary system (Bacaj & Shaham, 2007) and a repressible Q binary system (Wei, Potter, Luo & Shen, 2012). These developments will likely become more widely used for cell-specific expression in *C. elegans* in the near future.

2.5 Cell type-specific expression of transgenes in *Arabidopsis*

Aside from animal models, the most prominent species of plant utilised in molecular biology is *Arabidopsis thaliana*. Transgenic *Arabidopsis* have traditionally been generated by *Agrobacterium tumefaciens* T-DNA mediated transformation (Valvekens, Montagu & Van Lijsebettens, 1988; Hansen & Chilton, 1996). Characterised promoters can be fused to transgenes to allow cell type-specific expression, a method used for isolating the nuclei of specific cells in plants (Deal & Henikoff, 2010) (also see section 3.6).

A number of inducible binary systems are also available. These include a tissue-specific, alcohol-inducible system (Caddick et al., 1998; Deveaux et al., 2003). Here, the ethanol-regulated transcription factor, ALCR, is expressed in a subset of cells using specific promoters. Transgenes are placed under the control of the *alcA* promoter, which responds to ALCR only in the presence of ethanol. In addition, the GAL4/UAS system was adapted for use in *Arabidopsis* by Jim Haseloff in 1999 (Haseloff, 1999). This included GAL4 codon optimisation to allow efficient expression in plants. Similar to the enhancer trap screens performed in *Drosophila* (Manseau et al., 1997; Gates & Thummel, 2000; Mollereau et al., 2000; St Johnston, 2002), a GAL4 enhancer trap T-DNA vector was used to identify specific GAL4 lines that express in the lateral root of *Arabidopsis* (Laplaze et al., 2005).

Overall there are a wide variety of genetic systems in place for the cell-specific expression of transgenes, many of which span across the majority of model organisms. An intriguing possibility, which has surfaced in recent years with the advent of genome editing through the CRISPR/Cas9 system, is the ability to produce transgenic organisms from traditionally non-model species provided the genetic sequence is available. Using this technique, it is possible for researchers to produce tailored genomic manipulations to alter gene expression and/or function in the specific cell-types of interest opening many avenues for research (Harrison, Jenkins, O'Connor-Giles & Wildonger, 2014; Kistler, Voss hall & Matthews, 2015).

3. Profiling transcriptional activity and protein-DNA interactions by cell/nuclei isolation

Numerous methods exist for cell-specific profiling, all of which vary in their degree of accuracy, yield, and technical difficulty. In this section we will explore the various methods commonly used to obtain cell profiles through isolating individual cells or cell types from whole organisms or tissue.

3.1 Manual isolation

Perhaps the most obvious technique to select for individual cells or cell types is to manually identify and isolate the cells of interest. As expected, this method is often arduous, demands a great deal of technical expertise, and is time consuming. However, proper implementation can yield highly pure results and be employed to select rare subtypes (Okaty, Sugino & Nelson, 2011). Used predominantly in mice tissue, this method of selection has been employed since 2006 and enables the investigation of a variety of cellular profiling techniques, as entire cells are obtained (Sugino et al., 2006; Hempel, Sugino & Nelson, 2007).

Manual isolation traditionally takes advantage of tissue-specific fluorescent proteins (e.g. GFP) which, when under the control of a cell type-specific promoter, are expressed only in the cells of interest (Gold & Brand, 2012). When the organism is at the desired stage of development, the tissue is dissected, partially digested with a proteinase, and sufficiently diluted to obtain an appropriate amount of cells to be viewed manually. Once diluted, samples are suspended within a petri dish which can then be visualised using a fluorescent dissection microscope. The tagged cells are then easily identifiable and are extracted individually using an aspirator and micropipette (Egger, Gold & Brand, 2010; Gold & Brand, 2012). Subsequently isolated cells can be washed to improve the purity of the sample (Hempel et al., 2007; Okaty et al., 2011). Isolated cells can then be utilised immediately for *omic* profiling of specific cell types, or for individual cells given sufficient material is obtained.

As a highly effective method, manual isolation was initially utilised to identify the expression profiles of various neuronal subtypes in the mouse (Sugino et al., 2006). Through the technique's development, it has been used to characterise temporal transcriptome differences between groups of neurons within the central nervous system (CNS) (Okaty, Miller, Sugino, Hempel & Nelson, 2009). It has also proven successful in the fruit fly where it's been employed to assess mRNA expression both temporally and spatially (Egger et al., 2010; Bossing, Barros, Fischer, Russell & Shepherd, 2012), and furthermore, has been implemented without the use of genetically modified GFP expression, where mature neurons within *Drosophila* have been stained using the fluorescent dye DiI post-extraction (Bossing et al., 2012). This technique enables the use of manual isolation in non-model organisms whereby neuronal cell-specific profiling may be informative (Honig & Hume, 1989).

The manual method of cell sorting and isolation has been praised and criticised for its strengths, weaknesses, and contribution to the field. While it ensures low levels of contamination (<5% given adequate competency) and remains one of the cheapest ways in which to selectively isolate cells (Okaty et al., 2011), manual isolation is inherently technically demanding requiring a great deal of skill from the researcher, introducing potential human error, as well as time. This method therefore may be more appropriate for selecting rare cells (Hempel et al., 2007) while other more automated techniques could be more appealing and efficient when ascertaining other subpopulations.

3.2 Fluorescence-activated cell sorting (FACS)

One form of automatic cell-sorting available to researchers is fluorescence-activated cell sorting (FACS). FACS was first described in 1969 (Hulett, Bonner, Barrett & Herzenberg, 1969) by Leonard Herzenberg and a team of engineers at Stanford University who developed a machine that could detect fluorescent cells in a liquid stream of droplets (disrupted by a piezocrystal) and sort them by applying an electrical charge (see Figure 3A). Subsequently, with the incorporation of an argon laser, they had achieved a purity of approximately 50% (Julius, Masuda & Herzenberg, 1972; Lanier, 2014). It is now possible to achieve purities of greater than 98% using this method (Harzer, Berger, Conder, Schmauss & Knoblich, 2013).

FACS, like the Manual isolation method, makes use of cell-specific fluorescent markers but can also label the cells of interest using fluorescently-labelled antibodies. As dissociated cells are streamed through the flow cytometry platform, broken droplets (containing individual cells) are directed through a fluorometer which identifies individually-chosen characteristics of the cell (e.g. fluorescent intensity). Based upon the intensity of the specified characteristics, droplets are administered an appropriate charge, enabling their diversion towards a specified container (see Figure 3A) (Okaty et al., 2011). Although Herzenberg's first machine was based on a system which could sort cells based upon their volume at a flow rate of approximately 1000 cells/sec (Fulwyler, 1965), modern models can detect and charge droplets at a rate of approximately 3000 cells/sec as well as to sort cells based on multiple parameters including cell size and granularity (Zhu & Murthy, 2013).

With the speed and selective capacity of the FACS method, it is not surprising that it has been employed for a wide variety of investigations. It has easily been adapted for use with various model organisms including plants, worms, flies, fish and mice, and for isolating a wide range of tissues and cell types, from photoplasts to hematopoietic stem cells (Herzenberg et al., 2002; Birnbaum et al., 2005; Cahoy et al., 2008; Marsh, Minarcik, Campbell, Brooks-Kayal & Golden, 2008; Challen, Boles, Lin & Goodell, 2009; Berger et al., 2012; Gallardo & Behra, 2013; Spencer et al., 2014). The success and ease of this method has even led to researchers employing FACS to sort whole mutant live organisms (Fernandez et al., 2012). Furthermore, as FACS sorts cells through fluorescence, cells of interest can be labelled with fluorescently-tagged antibodies allowing for the investigation of fixed tissue from non-model organisms including humans (Atreya et al., 2000). Within the context of the CNS, FACS has been applied to investigate expression profiles of various neuronal cells, commonly in both *C. elegans* (Zhang et al., 2002; Colosimo et al., 2004) and mice (Buchstaller et al., 2004; Todman, Han & Herbison, 2005) where candidate genes for stem cell regulation, novel neurotransmitter receptors, and specification of certain neuronal sub-types have been identified (Molyneaux et al., 2009).

FACS is therefore widely applicable, provides excellent purity, and is a relatively high-throughput method, however, it can also have its drawbacks. For cells to be sorted, they first need to be dissociated from each other. This process can lead to the induction of stress responses (e.g. transcriptional and epigenetic changes) and even cell death, especially if cells are tightly coupled to one-another (Seidl, Knuechel & Kunz-Schughart, 1999; Mollet,

Godoy-Silva, Berdugo & Chalmers, 2008; Beliakova-Bethell et al., 2014). For situations involving cell culture or cells that are free in blood or haemolymph, this is less of a concern (Pruszek, Sonntag, Aung, Sanchez-Pernaute & Isacson, 2007). However, for many cell types that form tightly adhered connections to their neighbours or to those that possess non-classical shapes and projections, dissociation will undoubtedly induce undesirable gene expression. Mature neurons in the brain are an obvious example where FACS sorting is often too stressful as they both are densely interconnected and can possess axonal projections which can extend over ten times their cell body width (Arlotta et al., 2005; Lobo, Karsten, Gray, Geschwind & Yang, 2006; Heiman et al., 2008).

Due to their abnormal morphology, these cells can become fragmented and mRNA/DNA lost (Sanz et al., 2009; Gay, Karfilis, Miller, Doe & Stankunas, 2014). Although alterations can be made to the system to reduce these stresses on the cell, the nature of cell sorting through flow cytometry can be deadly to many cell types (Pruszek et al., 2007). Therefore, although FACS may be a desired option for those investigating cell-specific profiles of highly abundant and commonly-shaped cells, other methods can be utilised to investigate the characteristics of mature neurons, and other potentially complicated cell types, within the organism.

3.3 Immuno-panning (PAN)

FACS can be used to sort cells that aren't genetically labelled, by utilising antibodies that recognise specific antigens on the surface of the cells of interest. An alternative method that takes advantage of cell type-specific antigens is immunopanning (PAN) (Barres, Silverstein, Corey & Chun, 1988; Barres et al., 1992). As stated previously, the use of antibody staining allows researchers to use samples obtained from non-model organisms (provided antibodies for the cell-specific antigens are available), and thus opens the application of these methods.

To prepare samples for PAN, cells from the tissue are dissociated into a suspension and incubated for approximately 1 hour on a plate coated with the relevant antibody. The cells of interest bind to the antibody and the unwanted cells can be washed off (see Figure 4) (Lee, Chong, Tuck, Corey & Chan, 2013). Alternatively, it can be performed in a subtractive manner, i.e. to remove cells with the specific antigen and retain the cells that did not bind to the plate. With the specificity of the antibodies, PAN produces a relatively pure yield of cells, with contamination rates on par with both FACS and manual isolation ensuring its attractiveness as a cell-isolation method (Okaty et al., 2011).

Despite PAN's early development in the late 1980s (Barres et al., 1988), its uses have remained fairly limited within molecular biology. Initially utilised in rats to purify glial precursor cells, leading to the discovery that these precursors and their resulting oligodendrocytes require survival signals during neuro-development (Barres et al., 1992), the approach has now be used for cell isolation in mice and even humans, although remaining specific to cells within the CNS (Nieweg, Schaller & Pfrieger, 2009; Zhang et al., 2010; Ogawa, Tokumoto, Miyake & Nagamune, 2011; Foo, 2013). More recently, this method has been employed, together with FACS, to isolate cells for mRNA profiling of astrocytes, neurons, and oligodendrocytes (Cahoy et al., 2008). Interestingly, the profiles of

these cells suggested that the glial cells, astrocytes and oligodendrocytes are as different from each other as they are from neurons.

One of the drawbacks of the PAN method is that multiple plates with different antibodies often have to be used to ensure sufficient enrichment of the specific cell types (Barres et al., 1988; Barres et al., 1992; Cahoy et al., 2008). This can further contribute to the induction of cellular stress responses induced by the procedure, leading to undesired gene expression and confounding results (Gay et al., 2014). In addition, if there is no specific antigen expressed by your cell of interest (or those you wish to subtract) then it will not be possible to purify your cells using this technique, without using transfection or transgenic methods limiting its application.

3.4 Magnet-Activated Cell Sorting (MACS)

Yet another method that takes advantage of the specificity of antibodies is Magnet-Activated Cell-Sorting (MACS). Developed in 1990, MACS employs the use of magnetic-particles linked to antigen-specific antibodies to quickly and cheaply sort cells from full lysates (Miltenyi, Müller, Weichel & Radbruch, 1990).

For MACS, cells are dissociated and mixed in a solution. Magnetic particles are introduced which are coated in antibodies specific to antigens expressed on the surface of the cells of interest. A magnetic force is applied (magnetophoresis) which holds the cells of interest while the unattached cells are washed away (see Figure 3B). The magnetic force is then relieved and the cells left in solution are those of the desired cell type (Zhu & Murthy, 2013). Of course, if the desired cells possess natural magnetism (e.g. erythrocytes), antibodies do not need to be applied and cells can be sorted directly from serum (Safarik & Safariková, 1999).

As MACS can sort entire cell populations at once, unlike FACS which assess individual cellular-characteristics, MACS is a much quicker method. Furthermore, it is a much cheaper technique due to the inexpensive equipment required and can be undertaken at the lab bench, highlighting its accessibility (Schmitz et al., 1994; Zhu & Murthy, 2013). Given the cost and time benefits of this technique, it's relatively unsurprising that purity of the sample is reduced in comparison to FACS, reaching approximately 80% (Fong, Peh, Gauthaman & Bongso, 2009). More recently however, researchers utilising the MACS method for the sorting of bacterial cells have adapted the procedure with the addition of a second force. Termed Dielectrophoretic-MACS (iDMACS), cells are tagged with an additional particle which is susceptible to dielectrophoretic forces (i.e. the translational motion of charge-neutral matter caused by polarisation effects in non-uniform electric fields) as well as magnetic particles (Kim & Soh, 2009). Using this method, two cell types can be isolated at the same time from one sample, or both forces can be applied to the same cell to increase the purity of the retrieved cells (obtaining purities of approximately 95%). Alternatively, repeating MACS procedures (e.g. the 'three-step' MACS method) have been shown to increase the purity of the resulting sample (Lee & Lufkin, 2012).

MACS was initially developed in the mouse to investigate progenitors of blood cells (Schmitz et al., 1994), however now it has now been applied to a number of species and

investigations. Quickly adapted to use in human stem cells (Park, Moon & Kim, 2006; Fong et al., 2009), its importance in the selection of non-apoptotic spermatozoa was soon realised within assisted fertilisation (Makker, Agarwal & Sharma, 2008). To isolate and investigate germ-line cells and even neurons within *Drosophila*, a magnetic bead cell sorting method has been developed which works on the same principle as MACS (Wang, Starz-Gaiano, Bridges & Montell, 2008; Iyer, Iyer, Sulkowski & Cox, 2009). In the *Drosophila* system, the CD8-GFP transgene is often used to produce a cell-specific transmembrane tag which has high specificity, and commercially available antibodies for ease of isolation (Wang et al., 2008). Most recently however, the application has been successfully adapted for its use in zebrafish to sort neuronal subtypes where it has been used to generate neuronal-restricted progenitor cultures (Welzel, Seitz & Schuster, 2015).

While MACS has developed as a cheap, easy and quick method of cell sorting, it experiences a number of downfalls that limit its use. While purity is much lower than other methods such as FACS, PAN or manual isolation (Fong et al., 2009), mortality rates in cells are also higher than experienced with FACS due to the abrasiveness of the procedure (Yan et al., 2009). In addition, highly cell-specific antibodies need to be available for the cell of interest or the ectopically-expressed transgene which can be specifically introduced (Wang et al., 2008). Furthermore, post-sorted samples are retrieved with magnetic beads attached which can cause difficulties for purification and subsequent profiling techniques (Zhu & Murthy, 2013).

3.5 Laser Microdissection of Cells (LMD)

Laser Microdissection (LMD) enables the isolation of a single, or cluster of, cell(s) selected under microscope guidance, from a complex tissue section (either frozen or fixed) without the need for antibodies or genetic labels (Emmert-Buck et al., 1996; Nelson, Hempel & Sugino, 2006). The recovered cell(s) can then undergo molecular profiling, such as transcriptomics, translomics, ChIP, genome sequencing, liquid chromatography and proteomics (Scheidt et al., 2002; Vrana et al., 2009; Murphy et al., 2012; Yi et al., 2012; Amatori et al., 2014; Legres, Janin, Masselon & Bertheau, 2014).

There are a number of different methods which employ laser microdissection to isolate cells (Okaty et al., 2011). Laser capture microdissection (LCM) is the most common procedure, which involves overlaying a thermoplastic film on to the fixed or frozen tissue. While the cells are visualised under an inverted microscope, a low-power infrared (IR) laser is used to melt the thermoplastic film around the cells of interest, which are then bound to film. Peeling away the thermoplastic then separates the targeted cells from the rest of the sample (Curran & Murray, 2005; Espina et al., 2006). The versatility of this technique allows for many cells (up to several thousand) to be isolated from a single sample in this manner (Liu, 2010). A more modern form developed by Arcturus® utilises the application of an additional UV laser to cut around the cells of interest prior to melting the thermoplastic and is completely automated (see Figure 5A) (Murray, 2008). This procedure enables a more specific capture reducing rates of contamination by other cell types.

Laser-cutting microdissection is distinct from LCM (Schütze & Lahr, 1998; Murray, 2008). Here, cells are placed on a polyethylene naphthalate (PEN) foil slide where they are “drawn”

around and isolated by a narrow beam UV laser. These cells can then be captured by gravity (as in Leica AS LMD; see Figure 5B), or “catapulted” off the slide using the photonic force of a de-focused laser pulse (as in Zeiss PALM), avoiding the use of (and potential complications caused by) thermoplastic capture (see Figure 5C) (Legres et al., 2014). This method allows for the specific isolation of a large number of cells in a dense tissue while the gentler isolation employed by LCM is more suitable for the isolation of single or small groups of cells (Liu, 2010).

LMD was initially developed for the investigation of genomic irregularities in tumour cells (Emmert-Buck et al., 1996), including analysing the loss of heterozygosity (Bertheau et al., 2001), microsatellite sequences (Giuffrè et al., 2005) and point mutations (Coulbault et al., 2005). The techniques of LMD have now advanced to enable the profiling of individual cells in human tissue (Pietersen, Lim & Woo, 2009) and to obtain transcriptomes (through RNA-Seq and other methods) from a diversity of single cells (Tang, Lao & Surani, 2011). In addition, recent investigations have been able to isolate individual chromosome arms from this method to provide effective sequencing (Seifertova et al., 2013). Although intended to assess animal cells, LMD was quickly adapted for the isolation of a variety of plant tissues and individual cells (Kerk, Ceserani, Tausta, Sussex & Nelson, 2003; Day, Grossniklaus & Macknight, 2005; Schmidt, Schmid & Grossniklaus, 2012). Furthermore, although LMD techniques require tissue to be frozen or fixed, more recent methods allow for the investigation of live cultured cells which could remove some of the limitations of this procedure (Murray, 2008). LMD has also been important for the progression of neuroscience. In rodents, it has been utilised to assess the transcriptomic alterations between dopaminergic and non-dopaminergic neurons, as well as between pre- and post-stimulation (Backes & Hemby, 2003; Chung et al., 2005; Greene, Dingleline & Greenamyre, 2005; Yao et al., 2005).

Although LMD methods are not limited by the availability of antibodies or the adaptability of the organism to genetic manipulation, this method is time consuming, costly and susceptible to contamination (Nelson et al., 2006; Okaty et al., 2011). Furthermore, the tissue is predominantly fixed, frozen and/or stained prior to microdissection which can distort the expression of the individual cells, and abnormal cell shapes (such as dendritic neurons) may not be excised fully leading to loss of RNA (Srinivasan, Sedmak & Jewell, 2002; Sanz et al., 2009). Given these limitations, other methods of isolation are sometimes preferred.

3.6 Nuclei isolation - INTACT

The dissociation of tightly adhered cells from each other and their surrounding environment, as mentioned previously, is one of the technical limitations of the FACS method of cell sorting. The isolation of nuclei tagged in specific cell types (INTACT) technique overcomes this difficulty by allowing homogenisation of the tissue, followed by isolation of intact nuclei. Nuclei can then be assessed and profiled for genomic, transcriptomic or chromatin-profiling thus offering a great array of potential cell-specific information to the researcher (Henry, Davis, Picard & Eddy, 2012).

First demonstrated in plants (Deal & Henikoff, 2010), INTACT involves the coexpression of a nuclear envelope-targeted fusion protein that is a substrate for *in vivo* biotinylation, in combination with the *Escherichia coli* biotin ligase BirA. BirA biotinylates the biotin ligase recognition peptide (BLRP) in the fusion protein. This provides a very stringent method for affinity purification by streptavidin-coated magnetic beads in a process similar to MACS (see Figure 6A). In *Arabidopsis* and *C. elegans*, nuclear specificity is achieved by expressing the nuclear envelope-fusion protein from a cell type-specific promoter, whilst in *Drosophila* the GAL4/UAS system can be utilised (Henry et al., 2012; Steiner, Talbert, Kasinathan, Deal & Henikoff, 2012; Ma & Weake, 2014). Therefore in the fruit fly, nuclei can be tagged with specific genetically-encoded nuclear proteins expressed only in the cells of interest which adhere to antibody-bound magnetic beads, thus taking advantage of the GAL4/UAS libraries available to the field (Henry et al., 2012).

INTACT has initially been used to investigate expression profiles in muscle and mesodermal tissues both during adulthood and embryonic development (Deal & Henikoff, 2011; Steiner et al., 2012). With its adaptation to the fly, interest moved to the CNS where the transcriptome of neurons were obtained (Henry et al., 2012; Ma & Weake, 2014). The exemplified success of this method in a range of organisms has led to INTACT's adoption in a number of lab groups to investigate both the chromatin regulation and proteome of the nucleus (Amin et al., 2014; Bowman et al., 2014). These studies have contributed to identifying the characteristic chromatin structures of developmental gene clusters in *Drosophila* as well as uncovering the genealogy of vital cardiac proteins within vertebrates, respectively. The recent explorations presented here demonstrate the advantage of this technique and highlight its potential use for future investigations.

Although INTACT is a relatively cheap method (as non-specialised equipment is used) to isolate nuclei, there are a few limitations which must be realised. It can currently only be applied to organisms which can be genetically labelled with nuclear envelope-tags, while potential information held within the cytoplasm or other organelles of the cell is lost. This is important for neurons and other abnormally-structured cells where particular mRNAs are processed locally within the cell (Hirokawa, 2006). Furthermore the 'staining' process from the application of antibodies to the tissue and the stresses of homogenisation may disrupt cellular expression and subsequent transcriptome/proteome profiles.

3.7 Nuclei isolation – BiTS-ChIP

BiTS-ChIP (batch isolate tissue-specific chromatin for immunoprecipitation) is similar to INTACT, in that it involves the isolation of nuclei. However, it is particularly suited for ChIP experiments and eliminates the risk of inducing stress responses in the cells by fixing the tissue before nuclei isolation (Bonn et al., 2012a; Bonn et al., 2012b). Furthermore, it is possible to use BiTS-ChIP without having to first make a transgenic, as long as antibodies are available for a cell type-specific nuclear protein.

This method of nuclei-sorting incorporates tissue-fixation by aldehyde before nuclei are extracted. Depending on whether a fluorescent protein is expressed in the cells of interest, cells may need to be stained with appropriate antibodies bound to fluorescent reporters. Once the nuclei have been extracted and suitably labelled, they are subjected to FACS

whereby the nuclei are charged dependent on the fluorescence detected, and subsequently collected in the respective receptacle (see Figure 6B). Nuclei can then be assessed for chromatin structure, regulation and/or modifications (Bonn et al., 2012b).

BiTS-ChIP was developed in *Drosophila*, utilising the GAL4/UAS system to drive expression of a nuclear-localised fluorescent protein. Bonn and colleagues applied this method to profile RNA Pol II and histone modifications in mesodermal cells during embryonic development (Bonn et al., 2012a; Bonn et al., 2012b). They covalently cross-linked whole embryos at different time points and subsequently isolated the fluorescently-labelled mesoderm nuclei using FACS. In this study they achieved a purity of 97.4%. This method allowed them to identify a relationship between the histone code and the spatio-temporal activity of mesodermal enhancers which would have unlikely been found if assaying the whole embryo. The same group has more recently used BiTS-ChIP to identify that enhancer loops are active throughout embryonic development (Ghavi-Helm et al., 2014) and its potential use in zebrafish development has been noted (Bogdanovi , Fernández-Miñán, Tena, de la Calle-Mustienes & Gómez-Skarmeta, 2013).

Given the success of BiTS-ChIP as a method for obtaining chromatin profiles throughout development and beyond, it is surprising how little the method has been applied. However, as a more recent process of nuclei-isolation and sorting, the technique is beginning to take notice within the field and the associated advantages have begun to be recognised (Bonn et al., 2012b; Bogdanovi et al., 2013; Gisselbrecht et al., 2013; Kvon et al., 2014). Aside from its obvious perks, the BiTS-ChIP procedure still requires more specialised equipment than INTACT.

4. Profiling transcriptional activity and protein-DNA interactions without cell/nuclei isolation

As discussed in the previous section, many techniques have been developed to overcome the problem of cell-specific profiling by physically isolating the cells/nuclei-of-interest from the whole tissue/sample to obtain the desired material. However, the process of extracting, labelling, and sorting cells in these manners can lead to the application of physical stress (as well as others) which perturb the natural gene expression of the cell. A way to avoid these potential concerns is to tag the cells/proteins/nucleic acids of the organism *in vivo* so that profile analysis can be completed on the whole organism/tissue/sample but only the material from the cells of interest is retained. A number of methods are now available to obtain such information, the predominant techniques of which are presented within this section.

4.1 TU-tagging (transcriptome profiling)

The first method of non-isolation cell profiling we will discuss is TU-tagging. Based on a method to label newly transcribed RNA (Cleary, Meiering, Jan, Guymon & Boothroyd, 2005), TU-tagging introduced spatial regulation of mRNA transcript-tagging such that they can be obtained from predetermined cell types (Miller, Robinson, Cleary & Doe, 2009). Isolating and labelling the RNA of specific cells within the organism is a significant step-

forward, enabling researchers to determine transcriptome differences between specific subpopulations of cells.

In Cleary et al.'s (2005) ground-breaking paper, they described a novel method for tagging newly synthesised mRNA transcripts in the parasite *Toxoplasma gondii* through uracil phosphoribosyltransferase (UPRT). In this process, UPRT couples ribose-5-phosphate to uracil producing uridine monophosphate (UMP) which is then incorporated into the RNA, yielding 4-thiouracil (4-TU) (see Figure 7B). Acting as a substrate for UPRT, the subsequent product is additionally incorporated into the RNA leading to thio-substituted nucleotides which can be labelled through biotinylation and isolated from other RNA transcripts usually through streptavidin-coated magnetic beads. Resultant transcripts can be used to analyse synthesis and decay rates in various biological contexts (Cleary et al., 2005; Dölken et al., 2008; Rädle et al., 2013). This biotinylation procedure was then combined with the GAL4/UAS system in *Drosophila* allowing the transcriptome of specific cell types to be obtained, and was termed thiouracil-tagging or 'TU-tagging' (Miller et al., 2009). Furthermore, temporal specification in RNA tagging was achieved here by administering the 4-TU to the organisms through feeding.

The application of this process in flies has paved the way for tissue-specific use in other model organisms (Miller et al., 2009). As stated previously, the non-spatially-selective form of TU-tagging was utilised to investigate the synthesis and decay rate of mRNA in organisms and from mammalian cell culture (Cleary et al., 2005; Dölken et al., 2008; Rädle et al., 2013). Following its introduction to spatially-dependent drivers, TU-tagging has been used to analyse the differences between transcriptomes of specific cell types. As it was developed within the GAL4/UAS system of the fruit fly, it was initially utilised to assess transcriptome profiles of neuroblasts and optical tissue in *Drosophila*. These experiments have identified new micro RNAs (miRNAs) and transcriptional factors which regulate neuroblast proliferation as well as the activation state of the JAK/STAT pathway in tumour formation (Feng, Thomas & Wang, 2012; Lai, Miller, Robinson & Doe, 2012; Weng & Cohen, 2012). More recently, TU-tagging has been adopted into the mouse system which has obtained few but promising results (Gay et al., 2013; Gay et al., 2014). In this model, temporal specificity is achieved through injecting 4-TU into the organism or tissue of interest.

As this procedure requires no dissociation prior to labelling, the transcriptome obtained from TU-tagging avoids the disruptive effects applied through other methods of RNA isolation. However, this process can only currently be applied to model organisms and has been shown to be biased towards uracil-rich mRNA transcripts, although this can be corrected for during analysis (Miller et al., 2009). Furthermore, difficulties have been found in obtaining adequate amounts of material as fewer transcripts are generally identified, and amplification is weaker (Hupe, Li, Gertow Gillner, Adams & Stenman, 2014). In addition, the incorporation of TU into background mRNA has been found to be toxic in flies in some conditions, which may limit the application of this method (Thomas et al., 2012).

4.2 Poly(A) mRNA tagging (transcriptome profiling)

Another method of extracting RNA from specific cell types without physical sorting is Polyadenylated (A) mRNA tagging. Although providing similar results to TU-tagging, this procedure, developed by Roy et al. (2002), requires more tissue preparation in order to isolate the desired mRNA transcripts and thus is less attractive to researchers.

Poly(A) mRNA tagging involves driving a cell-specific FLAG-labelled poly(A)-binding protein (PABP), which when expressed, binds to the mRNA in that cell (see Figure 7A). When the total RNA is extracted, the tagged mRNA can be immunoprecipitated with FLAG antibodies, amplified and assayed by microarray or next-generation sequencing to determine the transcriptome profile (Roy, Stuart, Lund & Kim, 2002; Yang, Edenberg & Davis, 2005).

Although initially developed to investigate transcriptome profiles and gene clustering in *C. elegans* muscle cells (Roy et al., 2002), Poly(A) mRNA tagging has been used to investigate tumour gene expression in mammalian cell culture (Penalva, Burdick, Lin, Sutterluety & Keene, 2004). It also wasn't long before the procedure was adapted for assessing cell-specific profiles within the mature nervous system. Kunitomo et al. (2005) was the first to utilise the Poly(A) mRNA tagging system in the sensory neurons of *C. elegans*, which was soon followed by further studies (Kunitomo, Uesugi, Kohara & Iino, 2005; Von Stetina et al., 2007). With its continued success, the system was adapted to other model systems such as *Drosophila*, where it was employed to investigate the expression profiles of photoreceptor cells (Yang et al., 2005), and the mouse where the procedure has assisted in the identification of sleep-related genes in the brain (Maret et al., 2007). Furthermore, within the mouse, researchers have utilised this method of transcriptomics to ascertain the expression profiles of neurons infected with Venezuelan equine encephalitis virus *in vivo* (Konopka et al., 2007). More recently, Poly(A) mRNA tagging has been developed to explore the transcriptomics of individual cells opening up a wide potential for its use in a variety of biological systems and questions (Takayama, Faumont, Kunitomo, Lockery & Iino, 2010).

Despite the great potential which Poly(A) mRNA tagging offers to biologists, it still possesses inherent complications. The method employs immunoprecipitation to isolate and purify the tagged mRNA, which, in addition to giving rise to non-specific signal, can be abrasive to this unstable molecule, leading to its degradation. Additionally, this method is only applicable to adenylated mRNAs, limiting this procedure's use to isolate transcripts without poly-A tails.

4.3 TRAP/RiboTAG mRNA tagging (translatome profiling)

Translating ribosome affinity purification (TRAP) and RiboTagging are methods used to label ribosomes directly such that they can be pulled-down, and the bound mRNAs used for translatomics (Sanz et al., 2009; Gay et al., 2014). Like Poly(A) mRNA tagging, this method is a useful, non-intrusive way for isolating specific RNA populations but also has the advantage of enabling researchers to assess the translatome rather than the transcriptome of the cells of interest. This can provide researchers with a more relevant snapshot of the cellular environment (Dougherty, Schmidt, Nakajima & Heintz, 2010).

TRAP was developed in 2008 in the mouse (Heiman et al., 2008). In Heiman et al.'s paper, they describe a technique whereby a large subunit of ribosomes, RPL10a, is transgenically labelled with enhanced GFP (EGFP), and put under the control of cell-specific regulatory elements. When expressed, the labelled ribosomes can be isolated from the lysate through immunoprecipitation using EGFP-antibody-coated magnetic beads. These can then be isolated and the RNA purified for RNA-Seq, microarray, or q-PCR analysis (Heiman, Kulicke, Fenster, Greengard & Heintz, 2014; Hupe et al., 2014) (see Figure 7C). Although this procedure was published previously in plants, cell-specific expression was not introduced and thus a global translome was isolated (Zanetti, Chang, Gong, Galbraith & Bailey-Serres, 2005). This limitation provided an opportunity for Heiman and colleagues to develop the method further. Alongside the publication of TRAP, an additional publication by Doyle et al. described the production of 24 individual TRAP mouse lines to investigate the major cell types of the CNS (Doyle et al., 2008). Although a unique method for mRNA isolation, a number of limitations reduce TRAP's potential use to researchers. Primarily, these include the production of individual transgenic mouse lines for each cell type of interest (Kapeli & Yeo, 2012).

RiboTag was described later by Sanz et al. (2009) to circumvent these limitations. Based on the same principle, RiboTag takes advantage of the Cre/lox system in mice. One animal carrying a cell-specific driver upstream of the Cre recombinase gene can be crossed with a mouse possessing a *loxP*-flanked exon4 (the C-terminus) of the ribosomal gene RPL22 followed by a hemagglutinin (HA)-tagged version of the same gene (RPL22^{HA} allele). The resultant offspring possess both an RPL22^{HA} allele as well as a cell-specific driver such that the HA-tagged ribosomes are produced exclusively in the cells of interest, and the lysate can be immunoprecipitated with HA-antibody-coated magnetic beads (Sanz et al., 2009; Tallafuss, Washbourne & Postlethwait, 2014). Employing this method enables the researcher to take advantage of the available Cre lines available to obtain cell type-specific ribosome-tagged expression, as well as the ability to use commercially available (and cheaper) HA-antibodies (Kapeli & Yeo, 2012).

Both TRAP and RiboTag were initially utilised in the CNS of the mouse. As RiboTag makes use of the unique Cre/lox system developed within mice, it can be applied to a number of different tissues. This has been demonstrated by Gonzalez et al. who investigated the translome of tumorous gliomas through the specific introduction of Cre recombinase in affected cells. In addition, Sanz et al. investigated the profiles of male reproductive tissue (Sanz et al., 2013; Gonzalez et al., 2014). TRAP, on the other hand, is adaptable to a variety of other organisms. Since the procedure's description, it has now been adapted to plants, fruit flies, frogs, and most recently zebrafish where it has found applications within assessing the translomes of CNS cells (Mustroph, Juntawong & Bailey-Serres, 2009; Thomas et al., 2012; Watson et al., 2012; Tryon, Pisat, Johnson & Dougherty, 2013). Aside from easily genetically-modified animals, TRAP has found a use in determining the cellular profiles of Purkinje neuronal cells in rats whereby the tagged RPL10a-gene was introduced to the cells of interest through a viral vector and associated mRNA extracted as described previously (Kratz et al., 2014). Similarly, Ekstrand et al. utilised mice expressing cell-specific RPL10a linked to an anti-GFP camelid nanoparticle gene sequence. With the

introduction of GFP (through viral injection), the lysate can be immunoprecipitated with GFP-antibody-coated magnetic beads, and the ribosomes of neurons in specific neuronal networks are pulled-down for RNA analysis. (Ekstrand et al., 2014).

It is clear that both TRAP and RiboTag are promising tools for transcriptome investigation; for example, RiboTag studies in mice, and TRAP for assessing CNS tissue in multiple organisms (Heiman et al., 2014). Despite their proven usefulness within the field, there are a number of limitations to both of these methods. As both isolate the mRNA bound to ribosomes, neither can differentiate between mRNA from active or stalled ribosomes (Kapeli & Yeo, 2012). Furthermore, neither technique can detect non-coding RNAs (ncRNAs) or miRNAs which limits their application (Gay et al., 2014). However, a new method, termed miRAP, has been developed for use in mice for identifying changes in these RNA species (He et al., 2012). Moreover, TRAP has previously been criticised for its high levels of contamination compared to other forms of RNA isolation (Okaty et al., 2011) and has often been regarded to yield low levels of mRNA. However, optimisation of the method's protocol have been reported to improve these caveats (Heiman et al., 2014). Overall, both TRAP and RiboTag have proven to be useful methods for isolating and selecting cell-specific transcriptomes, especially within the mouse, however for models where genetic manipulation is not easy, other techniques may be more favourable.

4.4 Cell type-specific expression of epitope-tagged proteins (transcriptome and protein-DNA interactions)

An alternative method to investigate the transcriptome of specific cell types, as well as protein-DNA interactions, is the use of epitope-tagged proteins. These cellular profiling techniques take advantage of immunoprecipitation purification (using highly specific and well characterised antibodies), and cell-specific expression drivers available within model organisms.

Analogous to TRAP or RiboTag, protein epitope-tagging involves the formation of transgenes whereby a protein of interest is fused to an epitope tag such as EGFP. Cell type-specific expression can be driven using a binary expression system and can also be temporally controlled, using methods such as GAL80^{ts} or the quinacrine in the Q system, (see section 2.1). When the tagged-protein is expressed, it can be pulled-down from the lysate, and any bound or associated DNA/mRNAs can be profiled by high-throughput sequencing.

An example of such a method was introduced in the previous section undertaken by He et al. (2012) to investigate the miRNA present in mouse brains using miRAP. In their experiment, He and colleagues tagged Argonaute AGO2, a protein involved in the RNA-induced silencing complex (RISC), which binds miRNAs and their mRNA products as part of RNA interference (RNAi). Utilising the Cre/lox system, they crossed a cell-specific driver Cre⁺ mouse with one carrying an epitope-tagged AGO2 (tAGO2) gene with a *loxP*-flanked stop codon upstream. This ensured that only cells of interest expressed the tAGO2 protein, and only miRNAs from these cells would be isolated through immunoprecipitation using epitope-specific antibody-coated beads. He et al. applied this method to identify novel miRNAs that are expressed in glutamatergic and GABAergic neurons.

A more recent example of epitope-tagged proteins is chromatin affinity purification from specific cell types by ChIP (CAST-ChIP) (Schauer et al., 2013). Developed in *Drosophila*, this procedure involves labelling DNA or chromatin-binding proteins, such as RNA polymerase II (Pol II) or histone HA2.Z, with a GFP tag, putting the genetic construct under control of the Gal4/UAS system, and immunoprecipitating the lysate with GFP antibody-coated beads. The associated RNA and/or chromatin can then be profiled using various techniques.

4.5 Targeted DamID (TaDa) (transcriptome and protein-DNA interactions)

TaDa (Targeted DamID) is an adaption of the original DamID technique developed by Bas van Steensel and Stephen Henikoff (van Steensel & Henikoff, 2000; van Steensel, Delrow & Henikoff, 2001), which allows DamID to be utilised in a cell type-specific manner. The DamID system employs DNA adenine methylation to create 'footprints' within the genome. These footprints correspond to regions of the genome where the Dam-tagged protein has interacted with the DNA.

DamID (DNA adenine methyltransferase Identification) involves fusing an adenine methyltransferase from *E. coli* to a protein of interest. When the Dam-fusion protein is expressed in cell culture or a transgenic organism, the methyltransferase will leave a methylation mark at GATC sites wherever the protein associates with the genome. These methylated sites can be conveniently cut by the methyl-sensitive restriction endonuclease DpnI, and the fragments PCR amplified for profiling with whole genome microarrays or next-generation sequencing (Choksi et al., 2006; Luo, Shi & Baker, 2011; Wu & Yao, 2013) (see Figure 8). The key advantages of the DamID system over ChIP are that it does not require the use of antibodies, and the process handles only DNA, avoiding complications with unstable RNA or proteins.

The Dam enzyme itself is highly active and toxic at high levels. Presumably through saturation of GATC adenine methylation, and a disruption of gene regulatory and DNA replication mechanisms. Therefore for an effective DamID experiment, it is paramount to limit the levels of the Dam protein to almost homeopathic levels, such that they are undetectable by western blotting (Vogel, Peric-Hupkes & van Steensel, 2007). This, until recently, has been achieved though expressing the Dam-fused transgene from un-induced promoters to achieve low-level expression (van Steensel & Henikoff, 2000; Bianchi-Frias et al., 2004; Vogel et al., 2007). This method of regulating the Dam-fusion protein brings with it the disadvantage of global expression such that it is expressed in all cell types, all of the time. In order to use DamID for cell type-specific profiling of protein-DNA interactions, Southall et al. established a Targeted DamID system in *Drosophila*, utilising the GAL4/UAS binary expression system (see section 2.1) (Southall et al., 2013). This was achieved by drastically reducing the levels of Dam-fusion protein translated from the mRNA, using the phenomenon of ribosome re-initiation (Luukkonen, Tan & Schwartz, 1995; Child, Miller & Geballe, 1999; Van Blokland, Hoeksema, Siep, Otte & Verhees, 2011).

Targeted DamID was first applied to profile RNA Pol II occupancy, thereby giving a readout of transcription in specific cells *in vivo* (see Figure 8). Pol II occupancy was studied in two different neural stem cell populations in the developing *Drosophila* larval brain.

Comparison of the datasets identified previously known genes, as well as novel ones, which are enriched or are specific to the respective populations (Southall et al., 2013). This study also highlighted genes that possess paused Pol II in one cell type, whilst they are fully transcribed in the other. Here, TaDa profiling was controlled temporally using GAL80^{ts} (see section 2.1) for a window of 24 hours. Due to the methylation signature being an average of the binding events over that 24 hours, the resulting biological replicates are highly reproducible ($r=0.88$). This reproducibility facilitates the identification of genes that are differentially expressed between different cell types as well as those which have altered expression levels due to the mutant background, within a cell-specific manner.

The original DamID system has been used to study the interaction of transcription factors (Sun et al., 2003; Bianchi-Frias et al., 2004; Choksi et al., 2006; Southall & Brand, 2009; Wolfram, Southall, Brand & Baines, 2012; Southall, Davidson, Miller, Carr & Brand, 2014), chromatin modifiers (Sun et al., 2003; Vogel et al., 2006; Filion et al., 2010), lamin (Guelen et al., 2008; Kind et al., 2013), and components of the RNAi machinery (Woolcock, Gaidatzis, Punga & Buhler, 2010) with DNA. In addition, long-range chromatin interactions have also been investigated by tethering Dam to a single location in the genome (Cléard, Moshkin, Karch & Maeda, 2006; Bartkuhn & Renkawitz, 2008). Each of these applications can be adapted for cell type-specific profiling using TaDa. Due to the very low levels of adenine methylation in eukaryotes, DamID is, and has been, adaptable to multiple organisms, including *Drosophila* (Bianchi-Frias et al., 2004; Southall et al., 2013), *C. elegans* (Schuster et al., 2010), plants (Germann, Juul-Jensen, Letarnec & Gaudin, 2006), human cell lines (Vogel et al., 2006; Vogel et al., 2007; Guelen et al., 2008) and yeast (Woolcock et al., 2010) exemplifying its versatility as a molecular profiling method.

Despite its many uses, TaDa does have some disadvantages. These primarily include its resolution, which is limited by the frequency of GATC sites in the genome (median spacing between GATC fragments is 195bp in *Drosophila*), and its lack of strand specificity. While it also cannot be used to directly profile histone modifications, it can be used to profile the enzymes that edit these modifications which can provide important insights into chromatin structure and re-organisation.

5. Discussion

The importance of investigating gene regulatory mechanisms at a cell-specific level has been reflected in the drive and development of such techniques over the past two decades. Classical ‘whole cell’ methods such as FACS and LCM are being utilised as much as ever, and are useful to determine a variety of cellular properties from the transcriptome to the proteome. However, there has been an effort to design systems that do not require cell isolation, require less starting material, and/or are specialised for profiling aspects of the cellular-state. These include BiTS-ChIP, which is particularly suited for ChIP and investigating histone modifications, as it allows for fixing of the tissue before nuclei isolation and yields highly pure populations (Bonn et al., 2012a; Bonn et al., 2012b). TRAP (or RiboTag) has been developed for use in plants, *Drosophila* and mice, providing the opportunity to study the translome rather than the transcriptome serving a closer representation of the cellular environment (Zanetti et al., 2005; Sanz et al., 2009; Thomas et

al., 2012). The newly developed Targeted DamID technique (Southall et al., 2013) is likely to become popular amongst researchers due to its versatility, and ease to perform within the lab. TaDa possesses the ability to profile the interaction of any protein that comes into close proximity to the DNA (e.g. transcription factors, RNA polymerase II, lamins and chromatin modifiers), does not require the use of specific antibodies, and only requires the handling of DNA, minimising concerns regarding unstable RNAs or proteins.

It is clear that there is still much potential for the development and improvement of the techniques discussed here. Aside from TaDa, all other profiling methods avoiding cell isolation, require the pull-down of RNA, which results in contamination by non-specific transcripts (Tallafuss et al., 2014). This drawback is more of an issue when profiling small numbers of cells within a tissue. Any improvement that could reduce this non-specific contamination would represent a significant advance in the field. Alternatively, if there were a method (similar to TaDa in its mode of action) to reverse transcribe and amplify RNA derived only from specific cells (without the use of pull-downs), this indeed would be a powerful tool.

TaDa has scope to be improved and adapted for other uses. One of the limitations of this method is the resolution, as it relies on the presence of GATC sites, which can vary greatly in their distribution. A mutant variant of Dam (DamK9A) that methylates ATC sites (Horton, Liebert, Bekes, Jeltsch & Cheng, 2006) has been used to increase the resolution of DamID (Xiao, Roman-Sanchez & Moore, 2010). However, this method (DamIP) relies on pulling-down the methylated DNA fragments using an antibody that recognises methylated adenines. Although this does increase the resolution (approximately 4-fold), it is no longer as elegant, requiring a pull-down rather than using the methyl-sensitive restriction enzyme DpnI.

DamID has also been employed to investigate long-range chromatin interactions (Cléard, Karch & Maeda, 2014), which could be made cell type-specific by TaDa, either by inserting transcription factor binding sites at the locus of interest (Cléard et al., 2006). Bas van Steensel and colleagues have developed an ingenious system to visualise DNA that has been methylated by Dam in living cells (Kind et al., 2013). By using a fragment of DpnI fused to GFP, they visualised the interactions of DNA with the nuclear periphery before and after cell division. This again could be adapted for use in a cell-specific manner, enabling the dynamics of protein-DNA interactions to be observed in living tissues.

With the advent of induced-pluripotent stem cells (iPS) (Takahashi & Yamanaka, 2006) and CRISPR (Jinek et al., 2012), there will undoubtedly be a move towards CRISPR edited human iPS-derived culture models. For example, directed differentiation of iPS cells towards particular cell types/tissues has already been achieved for cortical neurons and retinas (Shi, Kirwan & Livesey, 2012; Zhong et al., 2014). CRISPR technology will allow researchers to knock-in tags or trap specific promoters, such that they can derive certain human tissues and subsequently profile specific cells within them. For example, a tagged ribosome could be inserted into the genome in such a manner that it is only expressed in the cells of interest, through the hijacking of a relevant promoter. One could then profile the transcriptome of specific human cells in close-to *in vivo* conditions. This type of approach

could provide more relevant information with respect to disease models, and also reduce the quantity of animal research required.

As Otsuki and colleagues have highlighted previously, a ‘cell type’ is often arbitrarily defined by the expression of one or more genes (Otsuki, Cheetham & Brand, 2014), when in fact they themselves can form a very heterogeneous population. For example, cholinergic neurons are comprised of cells with a diverse set of cellular morphologies and sensitivities to extracellular signals. That said, investigating and comparing broad classes of cell types, *in vivo*, is still in its infancy, and still holds a great potential for discovery. However, as technology and our knowledge progresses, there will be a shift towards profiling smaller, distinct populations, and even towards the single cell level. In some cases this has already begun, with the development of Fluidigm technology, which has been used to isolate single cells for RNA-seq studies (Shalek et al., 2013; Pollen et al., 2014). Although signal-to-noise is an issue with individual cell profiling (Grün, Kester & van Oudenaarden, 2014), many single cells can be profiled in parallel. Improvements to reduce technical noise, together with parallel sequencing of large numbers of cells simultaneously, will help overcome the issue of signal-to-noise, helping to provide a deeper level of insight into cellular heterogeneity within tissues.

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Cell/nuclei isolation

FACS (section 3.2)

Hulett et al., 1969
Herzenberg et al., 2002

Immunopanning (section 3.3)

Barres et al., 1988
Cahoy et al., 2008

MACS (section 3.4)

Miltenyi et al., 1990

Laser micro-dissection (section 3.5)

Emmert-Buck et al., 1996
Liu et al., 2010
Legres et al., 2014

INTACT (nuclei isolation) (section 3.6)

Deal & Henikoff, 2010
Henry et al., 2012
Steiner et al., 2012

BiTS-ChIP (nuclei isolation) (section 3.7)

Bonn, Zinzen, Giradot, et al., 2012
Bonn, Zinzen, Perez-Gonzalez, et al., 2012

Without cell/nuclei isolation

TU-tagging (section 4.1)

Miller et al., 2009
Gay et al., 2013

Poly-A binding protein pull-down (section 4.2)

Roy et al., 2002
Yang et al., 2005
Konopka et al., 2007

TRAP/RiboTag (section 4.3)

Zanetti et al., 2005
Sanz et al., 2009
Thomas et al., 2012

TaDa (section 4.5)

Southall et al., 2013

Figure 1. Overview of methods available for cell type-specific profiling

These techniques can be broadly categorised into two classes: Ones that require physical cell or nuclei isolation and ones that do not (Hulett et al., 1969; Barres et al., 1988; Miltenyi et al., 1990; Emmert-Buck et al., 1996; Herzenberg et al., 2002; Roy et al., 2002; Yang et al., 2005; Zanetti et al., 2005; Konopka et al., 2007; Cahoy et al., 2008; Sanz et al., 2009; Deal & Henikoff, 2010; Liu, 2010; Bonn et al., 2012a; Bonn et al., 2012b; Henry et al., 2012; Thomas et al., 2012; Southall et al., 2013; Legres et al., 2014).

Figure 2. Binary expression systems that can be used for cell-specific profiling

(A) Binary systems using a transcriptional activator. Shown here is the GAL4/UAS system (Brand & Perrimon, 1993). One transgenic organism, expressing GAL4 under the control of a specific promoter, is crossed to another possessing UAS sites upstream of a transgene. In the resulting progeny, GAL4 drives expression of the transgene in the cells of interest. (B) The Cre/lox system (Gu et al., 1993) relies on one parent that expresses the DNA recombinase Cre, crossing to another that has a *loxP*-flanked stop cassette between a ubiquitous promoter and the transgene. In the Cre-expressing cells of the progeny, the stop cassette will be removed, allowing the transgene to be expressed.

Figure 3. FACS and MACS

(A) Fluorescence activated cell sorting (FACS) allows sorting of cells based on a fluorescent signal (from transgenic expression of a fluorescent protein or through labelling the cells with a fluorescent antibody). The cell suspension is broken down into a stream of droplets, each containing an individual cell. Depending on the fluorescent signal detected, an electrical charge will change the path of the droplet to determine which receptacle the cell is deposited into (Hulett et al., 1969). (B) Magnetic activated cell sorting (MACS) involves labelling of specific cells with magnetic beads (using antibodies that recognise an endogenous or

transgenic cell-surface antigen). The cell suspension is then passed through a magnetic column, where the labelled cells are retained and the unlabelled cells are eluted (Miltenyi et al., 1990).

Figure 4. Immunopanning

Dissociated cells are incubated on plates that are coated with antibodies to specific cell-surface antigens. Cells that possess these antigens will adhere to the plate, whilst cells that do not, will be washed off (Barres et al., 1988; Barres et al., 1992).

Figure 5. Laser microdissection methods

(A) The Arcturus® laser capture microdissection system involves covering the layer of cells with a thermoplastic film. The cells of interest are first cut out with an UV laser, before an IR laser is used to adhere them to the film. The film can then be lifted, leaving the non-specific cells behind. (B) The Leica Laser Microdissection system uses an UV laser to cut around the cells, allowing them to fall into a collection tube. (C) The Zeiss PALM microdissection system also uses an UV laser to cut out the cells of interest but relies on a laser-generated photonic source to propel the cell sample into the appointed receptacle.

Figure 6. Nuclei isolation methods

(A) INTACT (isolation of nuclei-tagged in specific cell type) requires the expression of a nuclear envelope fusion protein and a biotin-protein ligase (BirA). BirA covalently links biotin to the nuclear envelope protein, allowing the specific cells to be pulled-down by streptavidin-coated beads (Deal & Henikoff, 2010). (B) For BiTS-ChIP (batch isolate tissue-specific chromatin for immunoprecipitation), the tissue is first fixed prior to nuclei isolation. The cells of interest express a nuclear-localised GFP, such that the nuclei can be sorted with FACS following tissue dissociation (Bonn et al., 2012b).

Figure 7. RNA pull-down methods

(A) Cell type-specific expression of an epitope-tagged poly-A binding protein allows the pull-down of polyadenylated mRNA (Roy et al., 2002). (B) TU-tagging involves the expression of uracil phosphoribosyltransferase (UPRT) in specific cells. When 4-thiouracil (4-TU) is administered to the organism, UPRT incorporates 4-TU into newly made mRNA. Labelled RNA is biotinylated and isolated using magnetic beads (Miller et al., 2009). (C) TRAP or RiboTag can be used to profile the transcriptome by pulling-down a tagged ribosome subunit (and its associated mRNA) from specific cells (Zanetti et al., 2005).

TaDa (Targeted DamID)

Figure 8. Targeted DamID

Targeted DamID (TaDa) allows the cell type-specific profiling of protein-DNA interactions by fusing the protein of interest to an adenine methyltransferase from *E. coli* (Southall et al., 2013). The Dam-fusion protein is expressed at very low levels in the cells of interest and leaves a methylation mark at the sites of interaction. Genomic DNA is then extracted and processed for analysis by next generation sequencing. RNA Pol II can be profiled by TaDa providing a readout of transcriptional activity.

Table 1

Significant advantages and limits of individual isolation methods. Techniques requiring physical isolation and dissociation of cells/ nuclei are highlighted in red, those which do not are shown in blue

Method	What's Isolated?	Brief Description	Advantages	Limitations	References
Manual	Entire Cells	Manual isolation by aspirating cells	High purity; Cheap	Time consuming; Technically demanding	Sugino et al., 2006; Hempel et al., 2007
FACS	Entire Cells	Automated isolation by fluorescence	Automated; High purity	Damages Cells; Expensive;	Hullett et al., 1969; Harzer et al., 2013
PAN	Entire Cells	Isolation by immunoprecipitation	Can use genetically unlabeled cells	Requires antibodies; Stresses cells	Barres et al., 1988 & 1992; Lee et al., 2013
MACS	Entire Cells	Isolation by magnetism	Rapid; Cheap	Low purity; High cell mortality	Miltenyi et al., 1990; Zhu & Murthy, 2013
LMD	Entire Cells	Isolation by laser-directed dissection	Can obtain rare cell types; Can use unlabeled cells	Low purity; Expensive; Time consuming	Emmert-Buck et al., 1996; Nelson et al., 2006
INTACT	Nuclei	Isolation by magnetism	Cheap; Ease of method	Requires antibodies; Ignores non-nucleic RNA	Deal & Henikoff, 2011; Henry et al., 2012
BITS-ChIP	Nuclei	Automated isolation by fluorescence	No fixation prior to isolation	Costly; Currently only available in <i>Drosophila</i>	Bonn et al., 2012a & b; Ghavi-Helm et al., 2014
TU-Tagging	Transcriptome	Tags mRNA with 4-TU for pull-down	Requires no cellular dissociation	Low RNA yields; Potentially toxic	Miller et al., 2009; Gay et al., 2013 & 2014
Poly(A) Tailing	Transcriptome	Tags mRNA with PABP for pull-down	Effective & specific isolation of whole RNA	Requires antibodies & immunoprecipitation	Roy et al., 2002; Yang et al., 2005
TRAP/RiboTag	Translatome	Tags ribosomes for pull-down & extracts mRNA	Obtains actively transcribed mRNAs	Unknown whether active or stalled ribosomes	Heiman et al., 2008; Sanz et al., 2009
Protein-Tagging	Bound RNA/DNA	Tags & pulls-down proteins which interact with DNA/RNA	Potential investigation of any DNA/RNA-binding protein	Requires purification of protein from DNA/RNA	He et al., 2012; Schauer et al., 2013
TaDa	Methylated (Tagged) DNA	Tags & extracts DNA where labelled-proteins interact	Rapid; Cheap; Avoids RNA & protein pull-downs; High yield	Limited resolution; Translatome unknown	Southall et al., 2013 & 2014