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A panel of recombinant monoclonal antibodies against zebrafish neural receptors and secreted proteins suitable for wholemount immunostaining

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ABSTRACT

Cell surface receptors and secreted proteins play important roles in neural recognition processes, but because their site of action can be a long distance from neuron cell bodies, antibodies that label these proteins are valuable to understand their function. The zebrafish embryo is a popular vertebrate model for neurobiology, but suffers from a paucity of validated antibody reagents. Here, we use the entire ectodomain of neural zebrafish cell surface or secreted proteins expressed in mammalian cells to select monoclonal antibodies to ten different antigens. The antibodies were characterised by Western blotting and the sensitivity of their epitopes to formalin fixation was determined. The rearranged antigen binding regions of the antibodies were amplified and cloned which enabled expression in a recombinant form from a single plasmid. All ten antibodies gave specific staining patterns within formalin-treated embryonic zebrafish brains, demonstrating that this generalised approach is particularly efficient to elicit antibodies that stain native antigen in fixed wholemount tissue. Finally, we show that additional tags can be easily added to the recombinant antibodies for convenient multiplex staining. The antibodies and the approaches described here will help to address the lack of well-defined antibody reagents in zebrafish research.

1. Introduction

The intercellular connectivity of neurons is critical for correct function, but detailed descriptions of neuronal wiring circuits in vertebrates are still lacking, and their functional outputs poorly understood [1]. Mapping neuronal networks is a challenging task because vast numbers of neurons are densely packed within the central nervous system, and their many processes often connect to neurons at anatomically distant sites [2]. Mechanistically, the development of neuronal networks is regulated by a series of attractive and repulsive cues mediated by factors that are secreted by, and displayed on the surface of cells within the nervous system [3]. The expression of these guidance molecules is highly regulated and identifying when and where they are expressed is important to obtain a molecular understanding of neuronal network development and function. While important progress has been made on the large scale documentation of gene expression patterns within the nervous system, the technique usually used – *in situ* hybridisation – often only identifies the soma of the neuron expressing the gene, and not their functional protein products. This is a particular limitation for cell surface and secreted factors whose products typically function at a distance away from the cell body of the neuron, making antibodies that work on fixed neuronal tissue valuable reagents.

The zebrafish is an important model vertebrate organism for neurobiology [4]. The *ex-utero* development of optically-translucent embryos, and a range of genetic tools provide a broad technical platform for neurobiology research [5]. One limitation of the zebrafish model, however, is the lack of high-quality antibody reagents that work on wholemount fixed tissue. While monoclonal antibodies for use as tissue or cellular markers can easily be raised using the "shotgun" method [6–8], their use is initially limited because the identity of the antigen is at first unknown. The paucity

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of antibody reagents against defined zebrafish antigens is particularly acute for cell surface and secreted proteins. One explanation is that the glycans generally present on zebrafish extracellular proteins are highly immunogenic in mammals – which are commonly used for raising antibodies – so that the elicited antibodies are often not protein-specific [9]. Also, extracellular proteins are usually modified by glycans and disulphide bonds which are not easily mimicked by chemically-synthesized peptides, which are often used as antigens for generating antibodies. Together, these factors make raising antibodies against zebrafish extracellular proteins a difficult process with uncertain outcomes.

Recently, we reported systematic and scalable methods for the selection and cloning of recombinant monoclonal antibodies [10–12]. Using a pooled immunisation approach, we demonstrated that up to five monoclonal antibodies could be selected in parallel and cloned into a single, convenient expression plasmid for distribution and storage. By using the entire ectodomains of zebrafish cell surface proteins expressed in mammalian cells as antigens, we show this approach is suitable for raising antibodies to neural receptors that work in wholemount fixed tissue. We also extend the functionality of our antibodies by adding additional protein tags to facilitate applications such as convenient multiplex staining.

2. Materials and methods

2.1. Antigen and antibody expression and purification

All proteins were expressed in mammalian cells as recombinant proteins as described [11]. The extracellular regions of zebrafish cell surface and secreted proteins which were previously used in protein interaction screens [13–15] were subcloned into a plasmid with C-terminal rat Cd4 domains 3 + 4, an enzymatically biotinylatable peptide and 6-His tags [16]. Biotinylation was achieved by cotransfecting a plasmid encoding a secreted *Escherichia coli* BirA enzyme [17], and proteins purified [18] before dialysing into PBS and storage at 4 °C until use. Recombinant antibodies were purified either using Protein G columns, or Ni-NTA Sepharose if 6-His-tagged (Invitrogen).

2.2. Antibody selection, screening and cloning

Monoclonal antibodies were raised and screened by microarray printing as described [11]. Amplification of both the rearranged antibody light and heavy chain was performed using total RNA extracted from ~10^6 hybridoma cells and both the amplified rearranged light and heavy antibody variable regions were recombined with an overlapping "linker" fragment by PCR and cloned into a single plasmid [10]. Plasmids encoding functional antibodies were identified by colony PCR and expression selection [10,11]. The plasmids encoding all recombinant antibodies can be obtained from Addgene [19].

2.3. Antibody validation by Western blotting and formalin fixation by ELISA

Western blotting was performed as described [18] using either non-reducing or reducing conditions. Proteins were blotted onto PVDF membranes (Amersham), blocked in 2% BSA and probed with ~10 μg/ml primary antibody for 1 h at room temperature or at 4 °C overnight. ELISAs were performed essentially as described [17] using 100 μl of purified recombinant antibody diluted in PBS/0.2% BSA. To test antibody epitopes for formalin fixation sensitivity, immobilised protein ectodomains were incubated for up to 120 min in 4% formalin and washed in PBS before the addition of the primary antibody.

2.4. Addition of alternative detection tags to recombinant antibodies

Sequences were added to the C-terminus of the IgG_1 heavy chain to permit enzymatic monobiotinylation and a 6-His-tag (Bio-6-His) to facilitate purification by IMAC [16], or a FLAG tag were added using standard molecular biology procedures.

2.5. Immunohistochemistry

The animal experiments described in this study were performed in accordance with local and national UK Home Office regulations. Zebrafish embryos were fixed with 4% formalin for 3 h at room temperature or 4 °C overnight, washed with PBT, and blocked (PBS, 10% goat serum, 0.6% Triton and 1% DMSO) for 1 h at room temperature. Purified recombinant antibody was diluted 1:1 in 1% goat serum, 0.6% Triton and incubated overnight at 4 °C. Embryos were washed in PBST and incubated with a secondary anti-mouse-HRP for 2 h at room temperature. Antibodies were detected with a TSA signal amplification kit (Invitrogen). For double labelling, Bio-6-His-tagged antibodies were detected with an anti-Bio-HRP (BioRad) followed by TCA. Subsequently, the second antigen was labelled with a FLAG-tagged antibody which was detected with an alkaline phosphatase coupled anti-FLAG-antibody and visualized with Fast Red (Invitrogen).

3. Results

3.1. Preparation of neural extracellular protein antigens

To maximise the probability of selecting antibodies that recognise neural receptor proteins in fixed tissue, we reasoned it was important to use antigens containing epitopes found on the native protein. Extracellular proteins are posttranslationally modified with glycans and disulphide bonds, and so we expressed the entire ectodomains as soluble recombinant proteins in mammalian cells which add these modifications. We selected ten zebrafish receptors from an existing protein library [13–15] which were expressed within the developing zebrafish nervous system as determined by *in situ* hybridisation [15]. The ectodomains were expressed with C-terminal tags that enabled enzymatic biotinylation and a 6-His tag for purification [16]. The purified proteins typically resolved as a single smear by SDS–PAGE centred on their expected mass, presumably due to the presence of multiple glycoforms (Fig. 1A).

3.2. Monoclonal antibody selection, cloning and initial validation

To efficiently select antibodies to multiple different antigens in parallel, we immunised mice with pools of proteins and, after hybridoma generation and screening, antibodies of interest were cloned into a single expression plasmid [10,12]. We found that plasmids encoding functional antibodies were more easily identified using a transfection and ELISA-based screening approach [11] rather than the sequencing method used previously [10]; antibodies were then expressed recombinantly and purified. We first demonstrated that all antibodies except one (anti-CD276) recognised their respective unreduced antigen whereas antibodies to Sema4c, Bcan and Lrrc3b did not recognise reduced antigen suggesting they recognise a conformational epitope (Fig. 1B). Because these antibodies will be used to detect antigens in fixed tissue, we next quantified the rate at which the immunoreactivity of each antibody for its antigen was lost upon formalin treatment. Eight out of the ten antibodies were insensitive to fixation, but the antibodies recognising Robo1 and IgSF21b proteins did show some sensitivity, informing us that these antibodies may lose immunoreactivity upon well-fixed tissue (Fig. 1C).

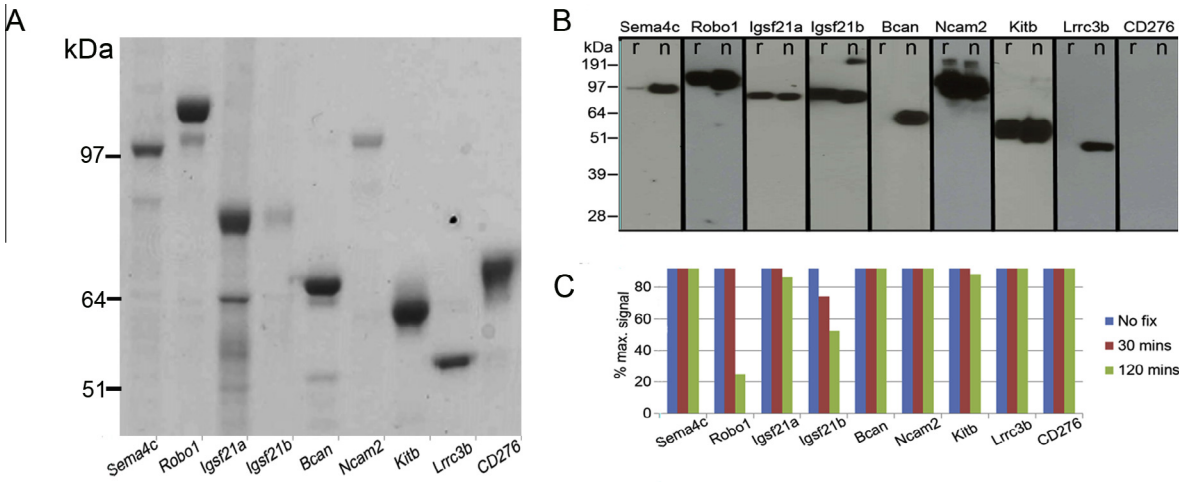

Fig. 1. Most antibodies work in Western blotting and recognise epitopes that are insensitive to formalin fixation. (A) Purified recombinant proteins were resolved at their expected mass by SDS–PAGE. (B) Antibodies were tested in Western blotting against both reduced (*r*) and non-reduced (*n*) antigens as indicated. The antibodies against Sema4c, Bcan, and Lrrc3b were sensitive to reduction of the protein suggesting they bind conformational epitopes, whereas those recognising Robo1, Igsf21a, Igsf21b, Ncam2 and Kitb recognised both reduced and non-reduced antigen. The antibody against CD276 repeatedly did not recognise antigen by Western blot. (C) The sensitivity of antibody epitopes to formalin fixation was evaluated. Antigens were fixed with 4% formalin for 0, 30 or 120 min and the fractional loss of immunoreactivity was quantified by ELISA. The majority of antibodies bound epitopes that were insensitive to formalin-fixation, although immunoreactivity of anti-Igsf21b- and anti-Robo1 antibodies decreased significantly after 120 min. Note that the antigen and antibody analysis for Kita is not shown.

3.3. Antibody staining patterns are consistent with localised transcript distribution in wholemount fixed tissue

We next used the antibodies to stain zebrafish embryos to determine if our approach led to the selection of antibodies that work on wholemount fixed tissue. Because a comprehensive expression atlas for each antibody is beyond the remit of this study, we focused on the developing zebrafish brain, and compared it to the corresponding *in situ* expression patterns [15]. An overall summary of the staining patterns is provided in Supplementary Table 1.

Three genes (*ncam2*, *igsf21a*, and *brevican*) are transcribed in discrete subpopulations of neurons within the developing brain, and the antibodies detected the corresponding protein products in the expected pattern. *ncam2* transcripts are expressed by neurons giving rise to the anterior (ac) and postoptic (poc) commissures at 30 h (Fig. 2A) [20]. As expected, the protein, which is implicated in neurite outgrowth [21], was located in these commissures at 2.5 dpf (Fig. 2A^{I}), and other tracts (Fig. 2A^{II}) including the dorso–ventral diencephalic tract (dvdt), the tracts of the posterior (tpc) and postoptic (tpoc) commissures and in the retina (Fig. 2A^{III}). Similarly, *igsf21a* is transcribed in the retina and telencephalic nuclei at 2.5 dpf (Fig. 2B); correspondingly, the antibody strongly stained the retina (arrows in Fig. 2B^{I}), and, more weakly, the telencephalon (asterisk in Fig. 2B^{I}) at 3 dpf. *brevican* is strongly transcribed in the ventral longitudinal tract (vlt) and discrete neurons throughout the forebrain (Fig. 2C), consistent with the antibody staining at 5 dpf within the vlt and forebrain tracts, including punctate staining in structures surrounding the tracts (arrow in Fig. 2C^{I}). These results suggested that our antibodies could specifically stain their antigens in fixed wholemount tissue.

3.4. Neural receptor genes transcribed throughput the developing brain have defined patterns of protein localisation

We have previously shown that the remaining genes have diffuse *in situ* staining pattern within the developing nervous system (Fig. 2D–J) [15]; however, the antibodies all labelled discrete brain structures. For example, Igsf21b is located along the supraoptic tract (arrow in Fig. 2D^{I}) and the prethalamus, tegmentum and the vlt (Fig. 2D^{II}). In the hindbrain, Igsf21b is expressed in a stripy pattern, most likely axons leaving the vlt (Fig. 2D^{III}). Kitb was localised in tracts and cells in the caudal brain region (Fig. 2E^{I}), and CD276 in the developing midbrain (Fig. 2F^{I}). Lrrc3b was strongly expressed on axons within the tract of the posterior commissure, the epiphysis, discrete regions in the telencephalon, hypothalamus and the dorsal midbrain (Fig. 2G^{I}). Dorsal views of the optic tectum showed Lrrc3b in neuropil-rich regions (arrow in Fig. 2G^{II}), often concentrated in punctate structures (asterisk in Fig. 2G^{III}) and more medially along axons positioned in cell body-dense regions (arrow in Fig. 2G^{III}). Robo is a guidance receptor and *robo1*-deficient zebrafish have defasciculated axons in the forebrain [22], consistent with this, anti-Robo1 labelled many forebrain commissures at 2 dpf, and some developing nuclei in the telencephalon. Robo1 is localised in a line of cells starting in the dorsal thalamus leading to dorsal parts of the tegmentum (arrow in Fig. 2H^{I}). At this stage, there was clear labelling in the posterior hypothalamus (arrowhead in Fig. 2H^{I}). Precursor photoreceptor cells are labelled in the epiphysis and axons projecting posteriorly from the epithalamus to the midbrain (asterisk in Fig. 2H^{I}). *sema4c* transcripts were expressed throughout the developing brain, but enriched in basal parts of the fore-, mid- and hindbrain. The Sema4c protein was detected in the anterior and postoptic commissures as well as in telencephalic nuclei and the olfactory epithelium (Fig. 2I). In the midbrain, Sema4c was located on the surface of cells at the dorsal and posterior edge of the two tectal lobes. Finally, the Kita receptor is located in neurons within the developing forebrain (arrow in Fig. 2J^{I}), midbrain (arrow in Fig. 2J^{II}) and in axon-rich ventral regions of the rostral brain (Fig. 2J^{III}).

3.5. Neural receptor proteins are detected in discrete subcellular localisations

Neural cell surface receptor proteins are sometimes concentrated within specific subcellular localisations such as synapses or even internal vesicles, providing clues to their functional role. Sema4c was detected at the apical site of the olfactory epithelium (arrowhead in Fig. 3A) consistent with its documented role in olfaction [23]. Within the neuroepithelium (arrow in Fig. 3A) and olfactory bulb (asterisk in Fig. 3A), Sema4c was detected within

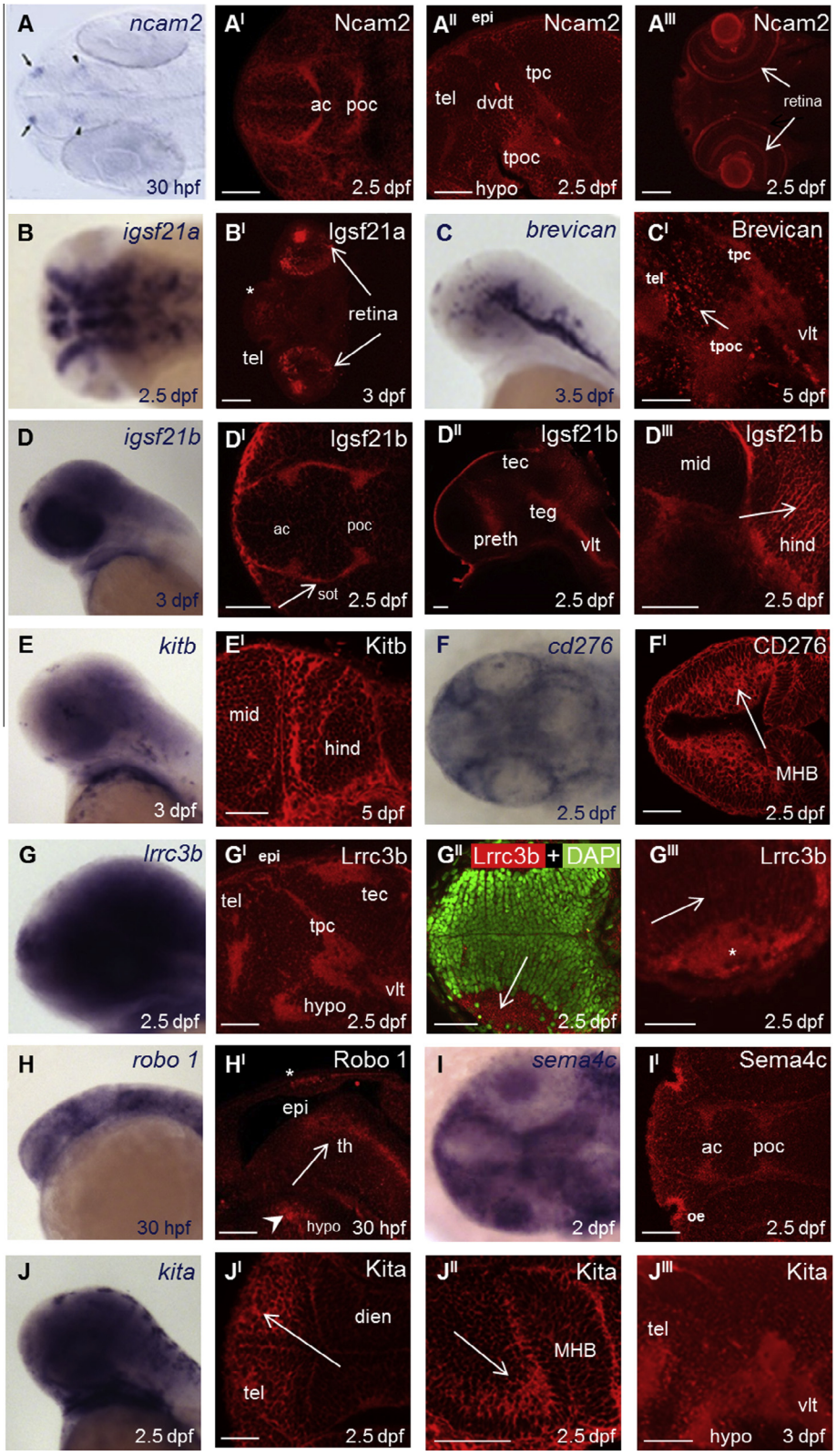

Fig. 2. Immunohistochemistry of the embryonic zebrafish brain with monoclonal antibodies to neural receptors and secreted proteins. The embryo stage and transcript/protein are indicated within each subpanel and staining patterns described within the main text. Transcript localisation is shown by wholemount *in situ* hybridisation as brightfield images; images of antibody staining are taken by confocal microscopy (red); nuclei are stained with DAPI in G^{II} (green). Note that some antibody staining, for example Igsf21a in the ventral diencephalon (B^{I}), is outside of the optical plane. Orientation of the embryos is always anterior to the left with ventral views in A, A^{I}, D^{I}, I^{I} and J^{I}; lateral in A^{II}, C, C^{I}, D, D^{II}, D^{III}, E, E^{I}, G, G^{I}, H, H^{I}, J and J^{III}; and dorsal in A^{III},B, B^{I}, F, F^{I}, G^{II}, G^{III}, I and J^{II}. Scale bars represent 100 μm in C^{I} and E^{I}, for all other images 50 μm. Abbreviations used in all figures are: ac = anterior commissure, dvdt = dorso–ventral diencephalic tract, epi = epithalamus, hind = hindbrain, hypo = hypothalamus, MHB = mid-hindbrain boundary, mid = midbrain, ob: olfactory bulb, oe = olfactory epithelium, poc = postoptic commissure, sot = supraoptic tract, tec = tectum, teg = tegmentum, tel = telencephalon, th = thalamus, tpc = tract of the posterior commissure, tpoc = tract of the postoptic commissure, vlt = ventral longitudinal tract, vnc = ventral nerve cord, hpf = hours post-fertilisation, dpf = days post-fertilisation.

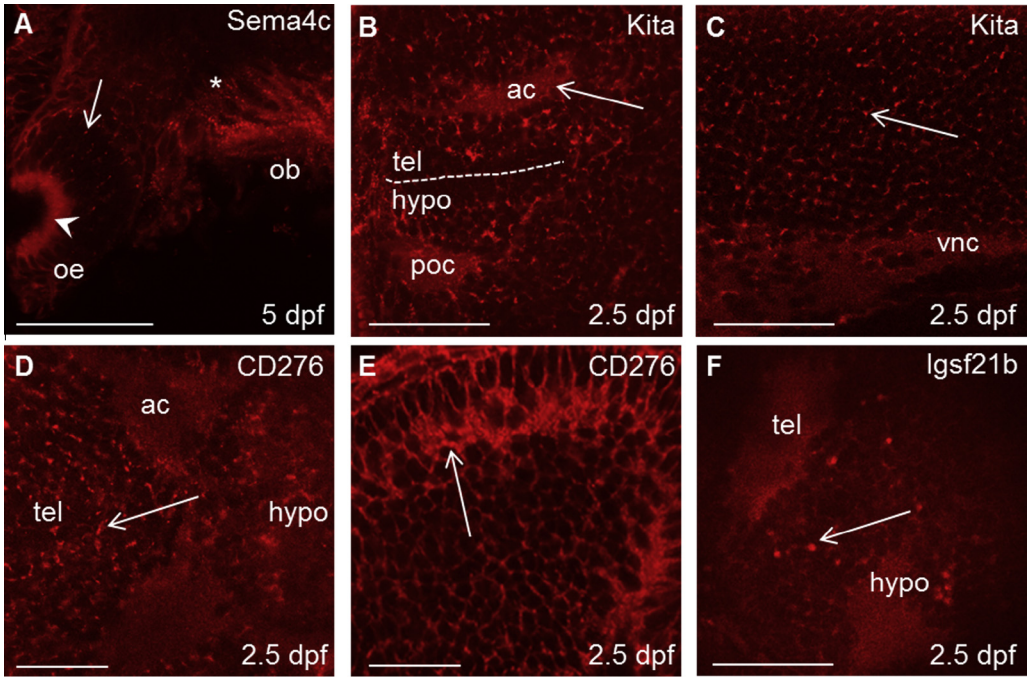

Fig. 3. Antibodies detect neural receptor proteins within discrete subcellular localisations. (A) Anti-Sema4c staining in the anterior neuroepithelium. (B) Anti-Kita labels the anterior (ac) and postoptic (poc) commissures and is also enriched at the periphery of cells (arrow). (C) Anti-Kita also stained axons of the ventral nerve cord (vnc) and the surface of hindbrain cells. Anti-CD276 staining in the developing midbrain (D), and tectum (E). (F) Anti-Igsf21b stained axon-rich regions in the anterior forebrain where it was often concentrated in discrete puncta (arrow). Views are anterior to the left and dorsal for A and E, lateral for B, C and F, ventral for D. Scale bars represent 100 μm in (A) and 50 μm in (B–F).

small puncta, consistent with the distribution of the murine orthologue in synaptic vesicles [24]. Kita was localised along axon tracts (arrow in Fig. 3B) and also the surface of the cell body (arrows in Fig. 3C); notably, neurons at the telencephalon-hypothalamus border were not stained with anti-Kita (Fig. 3B). CD276 was detected within the anterior commissure and was highly enriched on the surface of neurons in the telencephalon and hypothalamus (arrow in Fig. 3D). In the embryonic midbrain, we found the highest concentration of CD276 on the surface of posterior and lateral neurons, often concentrated in spots around a cell body (arrow in Fig. 3E). Finally, anti-Igsf21b stained distinct spots within the developing forebrain (arrow in Fig. 3F).

3.6. Convenient dual antibody labelling using interchangeable recombinant antibody tags

To facilitate multiplex staining applications, we constructed two plasmids containing C-terminal tags placed after the antibody heavy chain that contained either a biotinylatable peptide followed by a 6-His tag, or a FLAG tag (Fig. 4A). The regions encoding the anti-Lrrc3b and Sema4C antibodies were subcloned into the Bio-6-His-tagged and FLAG-tagged antibody plasmids respectively, expressed, purified, and used to simultaneously stain the rostral nervous system at 5 dpf (Fig. 4A). At the same site in the same embryo, anti-Lrrc3b detected with anti-Bio-HRP (Fig. 4B) and anti-Sema4c detected with an anti-FLAG-AP (Fig. 4C) gave distinct expression patterns when merged (Fig. 4D). In the area of the olfactory bulb, anti-Lrrc3b stained clustered puncta (arrow in Fig. 4B^{I}), or in similarly-sized rings (arrowhead in Fig. 4B^{I}) whereas anti-Sema4c stained with a grainy appearance (arrow in Fig. 4C^{I}) with some larger, brighter staining (arrowhead in Fig. 4C^{I}). At the junction of the telencephalon and diencephalon, there was an increase in the Lrrc3b-positive structures at the cellular periphery (arrow in Fig. 4B^{II}). Finally, Lrrc3b was again detected in subcellular puncta at the cellular periphery (arrow in Fig. 4B^{III}) whereas Sema4c was detected in a more diffuse pattern around cell bodies (arrows in Fig. 4C^{III}). Lrrc3b and Sema4c were colocalised at some sites (arrows in Fig. 4D^{III}). These plasmids enable rapid and convenient switching of protein tags on recombinant antibodies by simple subcloning procedures, and permit the simultaneous use of these antibodies in double or even triple antibody labelling experiments.

4. Discussion

The zebrafish is now a well-established model organism for neurobiology research and has proved particularly valuable for studying the very earliest stages of vertebrate neural development. Despite a diverse genetic toolkit, zebrafish research is hampered by the lack of good quality antibodies that work in fixed tissue. Here, we have shown that using the entire ectodomains of receptors expressed in mammalian cells – and therefore contain structurally-critical posttranslational modifications such as glycans and disulphide bonds – can elicit antibodies that stain native antigen within fixed embryos; indeed, all ten antibodies that we selected to different antigens worked in this application. Antigens that lack these modifications such as peptides are less likely to contain native epitopes that consequently result in antibodies that are unable to recognise the native protein. Our results suggest that the additional resource required to express proteins in mammalian cells is recouped by a greater probability of the antibody working on fixed tissue.

We have used this method to select a panel of ten monoclonal antibodies against zebrafish cell surface receptors expressed in the developing nervous system. Because of its experimental convenience, *in situ* hybridisation has been the major tool to determine where genes are transcribed during early zebrafish development [25]. In the three instances where the gene transcripts were localised by *in situ* hybridisation to identifiable subpopulations of neurons (*ncam2*, *igsf21a* and *brevican*), the corresponding protein gave the expected staining pattern, although typically along the

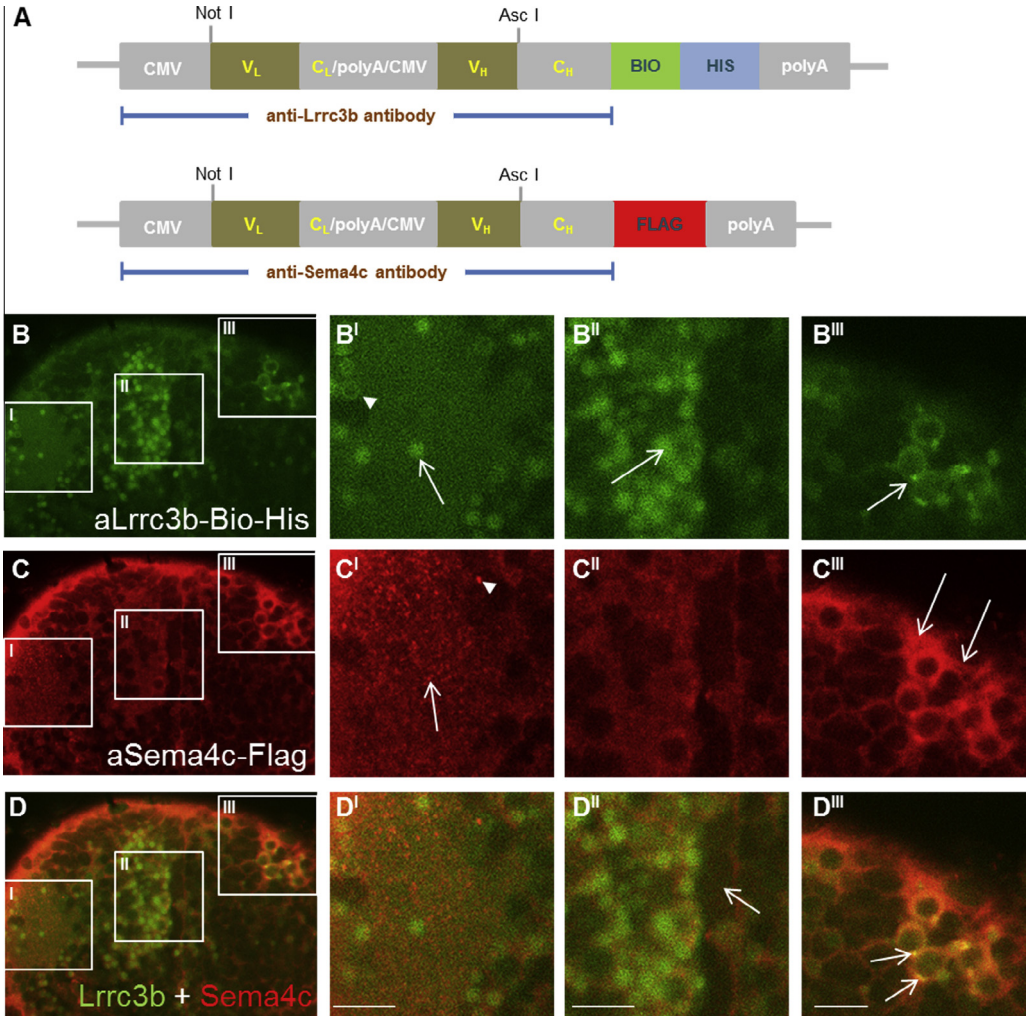

Fig. 4. The addition of different tags facilitates multiplex staining. (A) Schematics showing the addition of C-terminal Bio-6-His and FLAG tags to plasmids. The region encoding the specific antibodies are flanked by unique NotI and AscI sites facilitating antibody tag switching by subcloning. (B–D) The same 5 dpf embryo was simultaneously stained with both the biotin-tagged anti-Lrrc3b and FLAG-tagged anti-Sema4c antibodies. The staining of anti-Lrrc3b in green (B) and anti-Sema4c in red (C), together with the merged images (D) at the same site are shown. Staining patterns are described in the main text. Scale bars represent 50 μm.

projecting axons rather than the soma of the neuron as might be expected. Many genes encoding neural receptor proteins, however, are expressed throughout the developing brain using standard *in situ* protocols making hypotheses regarding their functional role challenging. In the seven remaining examples where transcripts were not localised, the antibody staining revealed the protein product was localised to specific brain regions, and in some examples, also had a defined subcellular localisation.

Recombinantly cloning antibodies has the advantage that they can be modified with additional functional groups relatively easily. Here, we have added biochemical tags which enable enzymatic monobiotinylation with an oligo-histidine tag, and also a FLAG tag. By using different tags, multiple primary and subsequently secondary antibodies can be simultaneously added to a staining reaction, cutting down the time required for dual labelling experiments significantly. It would be a relatively simple procedure to add additional tags to the recombinant antibodies to facilitate different detection methods or other functions.

In conclusion, we have demonstrated across a panel of ten zebrafish neural receptors an effective method that frequently leads to the selection of monoclonal antibodies that can be used to detect the native protein in fixed tissue. While the antibodies described here will be useful for neurobiology research, the general approach of expressing receptor ectodomains in mammalian cells could be similarly applied to receptors expressed in other tissues. Finally, the methods and antibodies described here will help to address the paucity of validated antibodies for zebrafish research.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.11.123.

References

[1] J.W. Lichtman, W. Denk, The big and the small: challenges of imaging the brain's circuits, Science 334 (2011) 618–623.
[2] J.L. Morgan, J.W. Lichtman, Why not connectomics?, Nat Methods 10 (2013) 494–500.
[3] M. O'Donnell, R.K. Chance, G.J. Bashaw, Axon growth and guidance: receptor regulation and signal transduction, Annu. Rev. Neurosci. 32 (2009) 383–412.

[4] R.W. Friedrich, G.A. Jacobson, P. Zhu, Circuit neuroscience in zebrafish, Curr. Biol. 20 (2010) R371–R381.
[5] Y.A. Pan, T. Freundlich, T.A. Weissman, D. Schoppik, X.C. Wang, S. Zimmerman, B. Ciruna, J.R. Sanes, J.W. Lichtman, A.F. Schier, Zebrabow: multispectral cell labeling for cell tracing and lineage analysis in zebrafish, Development 140 (2013) 2835–2846.
[6] B. Trevarrow, D.L. Marks, C.B. Kimmel, Organization of hindbrain segments in the zebrafish embryo, Neuron 4 (1990) 669–679.
[7] C. Crosnier, N. Vargesson, S. Gschmeissner, L. Ariza-McNaughton, A. Morrison, J. Lewis, Delta-Notch signalling controls commitment to a secretory fate in the zebrafish intestine, Development 132 (2005) 1093–1104.
[8] G. Deflorian, M. Cinquanta, C. Beretta, A. Venuto, C. Santoriello, D. Baldessari, F. Pezzimenti, M. Aliprandi, M. Mione, A. de Marco, Monoclonal antibodies isolated by large-scale screening are suitable for labeling adult zebrafish (Danio rerio) tissues and cell structures, J. Immunol. Methods 346 (2009) 9–17.
[9] D. Traver, B.H. Paw, K.D. Poss, W.T. Penberthy, S. Lin, L.I. Zon, Transplantation and *in vivo* imaging of multilineage engraftment in zebrafish bloodless mutants, Nat. Immunol. 4 (2003) 1238–1246.
[10] C. Crosnier, N. Staudt, G.J. Wright, A rapid and scalable method for selecting recombinant mouse monoclonal antibodies, BMC Biol. 8 (2010) 76.
[11] N. Staudt, N. Muller-Sienerth, G.J. Wright, Development of an antigen microarray for high throughput monoclonal antibody selection, Biochem. Biophys. Res. Commun. 445 (2014) 785–790.
[12] N. Muller-Sienerth, C. Crosnier, G.J. Wright, N. Staudt, Cloning of recombinant monoclonal antibodies from hybridomas in a single mammalian expression plasmid, Methods Mol. Biol. 1131 (2014) 229–240.
[13] K.M. Bushell, C. Sollner, B. Schuster-Boeckler, A. Bateman, G.J. Wright, Large-scale screening for novel low-affinity extracellular protein interactions, Genome Res. 18 (2008) 622–630.
[14] C. Sollner, G.J. Wright, A cell surface interaction network of neural leucine-rich repeat receptors, Genome Biol. 10 (2009) R99.
[15] S. Martin, C. Sollner, V. Charoensawan, B. Adryan, B. Thisse, C. Thisse, S. Teichmann, G.J. Wright, Construction of a large extracellular protein interaction network and its resolution by spatiotemporal expression profiling, Mol. Cell. Proteomics 9 (2010) 2654–2665.
[16] Y. Sun, M. Gallagher-Jones, C. Barker, G.J. Wright, A benchmarked protein microarray-based platform for the identification of novel low-affinity extracellular protein interactions, Anal. Biochem. 424 (2012) 45–53.
[17] J.S. Kerr, G.J. Wright, Avidity-based extracellular interaction screening (AVEXIS) for the scalable detection of low-affinity extracellular receptor-ligand interactions, J. Vis. Exp. (2012) e3881.
[18] S.J. Bartholdson, L.Y. Bustamante, C. Crosnier, S. Johnson, S. Lea, J.C. Rayner, G.J. Wright, Semaphorin-7A is an erythrocyte receptor for *P. falciparum* merozoite-specific TRAP homolog, MTRAP, PLoS Pathog. 8 (2012) e1003031.
[19] M. Herscovitch, E. Perkins, A. Baltus, M. Fan, Addgene provides an open forum for plasmid sharing, Nat. Biotechnol. 30 (2012) 316–317.
[20] T. Mizuno, M. Kawasaki, M. Nakahira, H. Kagamiyama, Y. Kikuchi, H. Okamoto, K. Mori, Y. Yoshihara, Molecular diversity in zebrafish NCAM family: three members with different VASE usage and distinct localization, Mol. Cell. Neurosci. 18 (2001) 119–130.
[21] M. Winther, V. Berezin, P.S. Walmod, NCAM2/OCAM/RNCAM: cell adhesion molecule with a role in neuronal compartmentalization, Int. J. Biochem. Cell Biol. 44 (2012) 441–446.
[22] C.A. Devine, B. Key, Robo-Slit interactions regulate longitudinal axon pathfinding in the embryonic vertebrate brain, Dev. Biol. 313 (2008) 371–383.
[23] L.C. Williams-Hogarth, A.C. Puche, C. Torrey, X. Cai, I. Song, A.L. Kolodkin, M.T. Shipley, G.V. Ronnett, Expression of semaphorins in developing and regenerating olfactory epithelium, J. Comp. Neurol. 423 (2000) 565–578.
[24] S. Inagaki, Y. Ohoka, H. Sugimoto, S. Fujioka, M. Amazaki, H. Kurinami, N. Miyazaki, M. Tohyama, T. Furuyama, Sema4c, a transmembrane semaphorin, interacts with a post-synaptic density protein, PSD-95, J. Biol. Chem. 276 (2001) 9174–9181.
[25] C. Thisse, B. Thisse, High-resolution *in situ* hybridization to whole-mount zebrafish embryos, Nat. Protoc. 3 (2008) 59–69.