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Generation, expression and utilization of single-domain antibodies for *in vivo* protein localization and manipulation in sea urchin embryos

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Abstract

Single-domain antibodies, also known as nanobodies, are small antigen-binding fragments (~15 kDa) that are derived from heavy chain only antibodies present in camelids (V_{HH}, from camels and llamas), and cartilaginous fishes (V_{NAR}, from sharks). Nanobody V-like domains are useful alternatives to conventional antibodies due to their small size, and high solubility and stability across many applications. In addition, phage display, ribosome display, and mRNA/cDNA display methods can be used for the efficient generation and optimization of binders *in vitro*. The resulting nanobodies can be genetically encoded, tagged, and expressed in cells for *in vivo* localization and functional studies of target proteins. Collectively, these properties make nanobodies ideal for use within echinoderm embryos. This chapter describes the optimization and imaging of genetically encoded nanobodies in the sea urchin embryo. Examples of live-cell antigen tagging (LCAT) and the manipulation of green fluorescent protein (GFP) are shown. We discuss the potentially transformative applications of nanobody technology for probing membrane protein trafficking, cytoskeleton re-organization, receptor signaling events, and gene regulation during echinoderm development.

1 Introduction

Deciphering embryogenesis requires specific molecular tools that can illuminate the abundance, subcellular localization, and complex molecular interactions of proteins. Traditional antibodies, also known as immunoglobulins (Ig), have been pivotal probes for a variety of imaging and biochemical analyses. Igs are naturally produced by the immune system to recognize specific peptide antigens. Antigen recognition takes place at the variable domains found in Ig heavy and light chains (V_H and V_L, respectively). Igs have two of each chain; one complete antibody molecule is comprised of four protein strands that are associated through disulfide bonds and non-covalent interactions. Specific binding of

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fluorescent or epitope-tagged antibodies enables the staining and signal amplification of antigens in a variety of immunohistochemistry-based assays. Antibodies are also essential for the purification of DNA-protein or protein-protein interactions isolated by immunoprecipitation. However, the tedious production process, complex protein structure, and large size (>150 kDa) of conventional antibodies limit their application for tracing or perturbing target antigens within living cells and whole organisms.

The discovery of unique, single-domain Igs in camelids (llamas, camels, alpacas; Hamers-Casterman et al., 1993) and sharks (Greenberg et al., 1995; Stanfield, Dooley, Flajnik, & Wilson, 2004) has revolutionized the application of antibodies for *in vivo* cell biology research (reviewed in Beghein & Gettemans, 2017; Dmitriev, Lutsenko, & Muyldermans, 2016; Harmansa & Affolter, 2018). Camelids and sharks produce heavy-chain only antibodies that utilize a single variable domain for complete antigen binding, termed V_{HH} in camelids and V_{NAR} in sharks. Isolated single variable domains are only ~15 kDa in size and thus designated “nanobodies” (Muyldermans, 2013). Nanobodies are small, highly soluble and stable molecules that can be recombinantly expressed in bacteria or yeast cells. This allows for an easily shared and perpetual supply of the probe. Notably, Nbs were used as pivotal chaperone molecules in Nobel Prize-winning protein crystallization experiments that identified G-protein coupled receptor (GPCR) conformations in different signaling states (Rasmussen et al., 2011).

What makes nanobodies most powerful is their ability to be genetically encoded and expressed within eukaryotic cells and whole organisms (Rothbauer et al., 2006). Nbs can be functionalized further by the addition of different effector domains (Table 1). For example, the fusion of fluorescent protein tags to Nbs (deemed “chromobodies”) generates stable probes for *in vivo* localization of target proteins. Importantly, Nbs in this form can be stored indefinitely as DNA fragments or full plasmid sequences. These types of probes have revolutionized cell and developmental biology research. In this chapter, we discuss how Nb technology is now being adapted to the sea urchin model system and its potential use in echinoderm research (Fig. 1; Table 2).

1.1 Structural basis of Nb recognition and stability in eukaryotic cells

Despite their difference in size, antibodies and nanobodies exhibit comparable binding affinities toward antigen (reviewed in Helma, Cardoso, Muyldermans, & Leonhardt, 2015). Antigen recognition in both full-length and single domain antibodies is mediated by complementarity determining regions (CDRs) present in the variable domains. There are three CDRs in each V chain, thus full-length Ig paired chains have six CDRs in total whereas single domain V_{HH}s have three. However, CDR1 and CDR3 of V_{HH} are longer than the conventional V_H CDRs, resulting in similar binding affinities and an antigen-interacting surface of 600–800 Å.. Nb binding is fast (~10^6 M^{-1} s^{-1}) and of high affinity (pM–nM) to its target antigen. The longer loops of V_{HH} also enable nanobodies to recognize cryptic or conformational epitopes that remain inaccessible to full-length antibodies.

The multimeric nature of full-length antibodies prevents robust expression for purification and utilization *in vivo*. The reducing environment of the cytoplasm prevents the formation of disulfide bridges that are required for the proper folding and stability of Ig molecules.

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Furthermore, without additional modifications, the functional fragments of single V_H and V_L exhibit low protein solubility and thermo-dynamic stability when produced in isolation from the multimeric full-length Ig protein. In contrast, the single V domains of Nbs have evolved to be extremely soluble in solution. They are routinely expressed in bacterial or eukaryotic cell systems with an epitope tag for affinity chromatography purification—Nb expression in these systems yields milligram quantities per liter of culture.

1.2 Applications of live-cell antigen tagging (LCAT) and protein manipulation

Pure Nb protein can be coupled with dyes or Alexa-Fluors. They are readily adapted into any conventional antibody-based method, such as immunoblotting, ELISA, pull-downs, or immunohistochemistry. The smaller size of Nbs allows for nanometer spatial resolution of targets in super resolution microscopy. When expressed *in vivo* as chromobodies, the small size of Nbs is also advantageous for traversing different cellular compartments and nuclear membranes. A summary of Nb approaches and potential LCAT applications in echinoderm research is presented in Tables 1 and 2, respectively, although these are not exhaustive lists (two excellent and detailed reviews of nanobody applications for imaging and developmental biology are Beghein & Gettemans, 2017; Harmansa & Affolter, 2018).

Nbs can be designed against two types of antigens: (1) widely utilized “tags,” such as fluorescent proteins or peptide epitopes, and (2) endogenous proteins. Each target has its advantages and disadvantages depending on the model system and experimental question at hand. Nbs of either persuasion can be delivered into cells or animals by genomic engineering; the Nb cDNA sequences are placed under the control of cell-specific, inducible, or ubiquitous reporter sequences (Panza et al., 2015; Rothbauer et al., 2006; Yamagata & Sanes, 2018). In model organisms lacking established transgenic lines like the sea urchin, *in vivo* Nb expression can be easily encoded in F_0 by using mRNA overexpression (Fig. 1–2), or by the mosaic integration (Rast, 2000) of reporter-based Nb transgenes. Select highlights of developmental biology applications across model systems are discussed below. Impacts for the sea urchin model system are discussed in Section 5.

1.2.1 Tag-binder applications: GFP-binding Nbs for tracing and perturbing tagged proteins of interest

—Some of the most versatile Nbs created to date are the GFP-binding Nbs and chromobodies. A series of GFP-binding Nbs were originally generated to bind GFP to improve super-resolution microscopy (Rothbauer et al., 2006). A handful of GFP-binding proteins (GBPs) were mutated to modulate the spectral properties of GFP and thus act as GFP-”minimizers” or “enhancers” (Kirchhofer et al., 2010). These GBPs shift the absorption spectrum of GFP such that florescent intensity is reduced or increased.

Further functionalization of GBPs has expanded their experimental scope for dissecting protein localization, signaling pathways, and even morphogen gradients. GBPs fused with subcellular localization signals can be used to identify protein-protein interactions with tagged bait, or to mislocalize target GFP-tagged proteins (reviewed in Harmansa & Affolter, 2018). The GrabFP system (Harmansa et al., 2017) redirects target proteins to set positions within the apical-basal axis of epithelial cells, for example. A similar “morphotrap” method

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couples GBP nanobodies to the extracellular surface of the cell, where they act to immobilize secreted GFP-tagged bait (Harmansa et al., 2015). This approach was elegantly used to decipher the role of BMP2/4 homolog gradients in *Drosophila* wing disc patterning. The most recent adaptation of GBPs for developmental biology is LlamaTags (Bothma et al., 2018). These Nbs were designed to visualize the rapid nuclear cycling of transcription factors (TFs) during *Drosophila* development.

Other GBP derivatives are designed to reduce or eliminate protein activity. One approach traps target proteins from maturing out of the ER (B€oldicke, 2017). In the deGradFP system, GFP-tagged targets are instead recruited for proteosomal degradation, via E3-ubiquitin ligases fused to the GBP Nb (Caussinus et al., 2012; Shin et al., 2015).

1.2.2 Endogenous-binders: Illuminating the cytoskeleton and cell cycle

One of the first characterizations of endogenous binding Nbs in a developmental system was done in zebrafish by the Rothbauer group (Panza et al., 2015). Transgenic zebrafish were generated that expressed anti-actin and anti-PCNA chromobodies under the control of an *hsp70l* promoter. Upon heat shock, Nb binding and antigen localization occurred through a specific, rapid association/disassociation mode of antigen-binding, similar to that of LifeAct probes (Riedl et al., 2008). Intracellular expression of the chromobodies enabled complete localization of their respective antigens across a variety of cell types and developmental stages, without toxic defects. It is hypothe-sized that the constant reversibility in binding minimized potential negative interference on target protein function. In contrast, inhibitory anti-actin Nbs have been evolved to bind to a conserved actin capping protein, CapG, to blunt cell migration events *in vitro* and *in vivo* (Van Impe et al., 2013).

Overall, because of their small size and rapid binding kinetics, most Nbs do not interfere with endogenous target protein function, unless they have been specifically mutated or selected to do so. Nonetheless, when using these probes for LCAT, careful assessments are necessary to ensure that chromobody binding to endogenous targets does not interfere with protein function. Comparisons of chromobody expression to different types of loss-of-function phenotypes are important controls in this context. Photobleaching experiments can also help determine if chromobody binding occurs with high on/off rates or appears more stable, the latter being more likely to lend inhibitory effects.

1.3 Applying Nb technology to the echinoderm model system

Nanobody technology enables *in vivo* experiments that probe most aspects of developmental biological inquiry. In this context, echinoderm eggs, embryos, and larvae are incredibly amenable to Nb expression and utilization. We present here an overview of utilizing Nbs in living *Strongylocentrotus purpuratus* embryos. The protocols for generating candidate binders from naïve, activated, or synthetically derived nanobody libraries are summarized for common principals, and covered in greater detail in the references therein.

For the researcher who seeks to use one of the readily available commercial nanobodies, we emphasize the procedures and controls necessary to clone and express validated Nb sequences as live biosensors in the sea urchin embryo. Our primary example is the live-cell antigen tagging and manipulation of GFP fluorescence in blastulae using GFP-binding

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nanobodies from the Rothbauer group. We envision that encodable Nbs will be equally amenable for illuminating endogenous targets. Future applications will enable elegant protein perturbation and gene-editing approaches, particularly as the CRISPR/Cas9 gene-editing technology advances in echinoderms (Tables 1 and 2).

2 General protocols for nanobody generation, recombinant evolution, and purification

Nanobodies from animals are generated and optimized from a variety of sources using standard molecular biology techniques (Fig. 1–1). Briefly, target antigen is expressed at high levels in yeast or bacteria and purified using affinity and size-exclusion chromatographies. To generate specific libraries, camelids or sharks are immunized with the purified antigen. Following an appropriate immune response, mRNA is isolated from the animal's blood cells and reverse transcribed into libraries of $>1 \times 10^5$ candidate V_Hs. The cDNA library is adapted into expression systems for phage-, yeast-, ribosome-, or mRNA/cDNA-display based methods of Nb panning against immobilized purified antigen. Display methods share two common principles: (1) large V_H libraries are screened by coupling cDNA sequence to its cognate peptide, and (2) multiple cycles of selection enable enrichment of specific binders against the antigen of interest. Selected Nbs are then purified in milligram quantities by standard molecular techniques in bacteria or yeast.

The primary bottlenecks for adopting nanobodies to the bench remain animal immunization and library production. However, immunization and library screening services are offered by several companies (see Appendix) and are often quite successful. Nonetheless, this route remains expensive and time-consuming, on the order of several months. Naïve animal libraries offer a faster alternative, albeit libraries must be derived from a much larger pool of blood samples to ensure the retrieval of high-affinity binders from less-diverse immunocytes.

In contrast, semisynthetic or synthetic libraries can circumvent the time and cost issues associated with animal-based libraries, down to only a few weeks. The synthetic approach couples peptides encoded from 10^5 to 10^{15} invariant sequences with traditional display technologies and *in vitro* molecular evolution procedures (Adams & Sidhu, 2014; Wada, 2013). The platform for such libraries is usually derived from consensus llama Ig gene sequences or humanized V_{HH} scaffolds that have been subjected to calculated variation of their CDRs. The library scaffolds are also optimized for strong intracellular expression and high stability of candidate Nb peptides. As long as a researcher has purified antigen of interest, it is possible to screen and evolve binders from synthetic libraries. Such libraries are available from several companies (Appendix) or have been generated by independent labs (Fridy et al., 2014; Moutel et al., 2016). Of note, protocols for a fully *in vitro* synthetic nanobody discovery platform, based on yeast surface display, are now available open source through the Kruse Lab (McMahon et al., 2018).

2.1 Evolution of candidate binders: mRNA/cDNA display methods

Regardless of the starting library source, display methods are designed to select Nbs specific to the target antigen. Cell-based methods can be limited by transformation efficiencies and

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plagued by contamination. Fully *in vitro* mRNA/cDNA display circumvents these issues. In this method, puromycin-based ligation technology permits the fusion of candidate binder cDNA with cognate mRNA and peptide (Fig. 1–1B; Roberts & Szostak, 1997). mRNA/cDNA display also offers higher stringency than traditional ribosome-based methods, especially for selection against membrane protein antigens (Doshi et al., 2015). Only small amounts of material are required for Nb evolution using this method—a total of <20μg of mRNA (~5μg/round of display) and <10μg of purified target antigen (~2μg/round of display).

1. Nb cDNA template library is *in vitro* transcribed into mRNA, purified with magnetic OligodT$_{25}$ beads (New England Biolabs), and checked for quality.
2. Purified mRNA is ligated to a puromycin linker.
3. Ligated mRNA is *in vitro* translated to protein. Puromycin linkage during translation forms a covalent mRNA-peptide fusion.
4. Fusions are re-purified with OligodT$_{25}$ beads and reverse-transcribed to re-synthesize template cDNA.
5. The mRNA/cDNA-Nb complex is exposed to the antigen of interest immobilized on an affinity matrix, such as Ni-NTA magnetic beads (Qiagen).
6. The matrix is washed, and the mRNA/cDNA-Nb complex is eluted.
7. PCR of eluted mRNA is performed to retrieve full-length Nb-encoding amplicons. Error-prone polymerases or molecular evolution methods can be used at this step to generate higher affinity cDNAs. Positive binders are reintroduced into the display cycle.

2.2 Purification of specific binders

1. Following iterative rounds of bench-top selection, final candidate Nb PCR products are cloned into *Escherichia coli* Top10 cells (Life Technologies), sequenced, and sub-cloned into vectors for large-scale overexpression and isolation in *E. coli* or eukaryotic cells.
2. Nb protein is expressed and purified using affinity and size-exclusion chromatographies.
3. Purified Nbs are ranked by binding affinity for detergent-purified target antigen, as measured by surface plasmon resonance (SPR).

2.3 Commercially and publicly available nanobodies

Depending on the experimental approach, pre-existing Nbs against common cell and molecular targets (such as GFP, RFP, F-Actin, or cell cycle proteins; Table 1) may be more amenable and cost-effective than deriving entirely new Nb libraries or evolving novel binders from synthetic libraries. Validated nanobody sequences can be found in publicly available protein or Nb-specific databases (Zuo et al., 2017), shared upon request from lab groups, or can be purchased as plasmids from Addgene or individual companies (Appendix).

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3 Protocols for cloning and synthesis of fluorescent-tagged Nbs for mRNA overexpression *in vivo*

Nb cDNA sequences can be easily cloned into expression vectors that enable the adaptation of nanobodies for use *in vivo*. In the sea urchin, fluorescent protein (FP)-tagged nanobody mRNA can be overexpressed in early embryos to validate target specificity and to localize endogenous proteins of interest throughout development (Fig. 1–2; Campanale et al., 2014; Gökirmak et al., 2012; Lepage & Gache, 2004). These methods are revisited in greater detail elsewhere in this volume. The Hamdoun Lab has constructed four FP tag variants (eGFP, YFP, mCherry and CFP) of the pCS2 + vector system (named pCS2 + 8-*N*-FP and pCS2 + 8-*C*-FP; Fig. 1–2) to enable the generation of stable mRNAs encoding N- or C-terminal fusion proteins (Gökirmak et al., 2012). The multicolor protein expression kit is available from Addgene (www.addgene.org). To boost Nb signal, additional FP tags can be cloned into vectors to generate 2X or 3X-FP tagged fusion proteins. The protocols for cloning GFP-binding Nb cDNAs into PCS2 + 8 vectors for LCAT applications are described below. We find great success with the InFusion cloning kit from Clontech. Specific resources and online tools are provided in Appendix.

3.1 Design positional cloning of Nb cDNA into PCS2 + 8 vectors for generating fusion Nbs

1. Design primers to Nb sequence, with overhang restriction enzyme sites. For example, for a C-terminal fusion Nb, *Asc*I and *Pac*I sites were used to insert a GBP4-Nb sequence directly upstream of the FP tag in the mCherry vector (Figs. 1–2 and 2).
    a. We have had the best experience with C-terminal Nb fusions. When designing C-terminal fusions, ensure that the Nb sequence does not contain a termination codon. When designing N-terminal fusions, the Nb sequence must contain the stop codon.
2. Manually inspect the predicted fusion protein product. This can be done within open source plasmid software or by using online tools. Check that the Nb protein sequence will remain in frame with the FP coding sequence after restriction sites have been added to the fragments.
3. Re-design of primers may be necessary; usually the removal or addition of a single nucleotide suffices.

3.2 Amplify Nb sequence and clone into PCS2 + 8 vectors

Depending on your Nb target, template may come from a cDNA library of screened Nbs, or from commercially or publicly available plasmid sources. PCR amplification is routinely used to subclone the Nb sequence into the expression vector; Nbs range from 350 to 500 bp in size. Alternatively, IDT Geneblocks of Nb sequence plus cloning overhangs can be synthesized directly from IDT.

1. Amplify template using a high-fidelity DNA polymerase. We routinely use Phusion or Q5 (New England Biolabs).

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2. Check for a single band by agarose gel electrophoresis, and gel extract the PCR product (Qiagen PCR and Gel clean up kits work well).
   a. For InFusion cloning (Takara Bio) reactions, proceed directly to cloning after clean up.
   b. For positional cloning, digest the insert with the appropriate restriction enzymes.
3. Calculate a 2:1 insert:vector ligation reaction. We have found that ligation reaction volumes can be cut in half to extend the reagents with no significant decline in yield. Molarity calculators are provided in the online InFusion tools.
   a. Incubate the InFusion ligation reaction at 50°C for 15 min. Meanwhile, prepare a water bath or incubator for 42°C.
   b. Add 1.25 μL of the ligation reaction to 25 μL Stellar (New England Biolabs) chemically competent cells inside a 14 mL round bottom culture tube.
   c. Incubate for 30 min on ice. Meanwhile, pre-warm S.O.C media (New England Biolabs) and LB ampicillin media plates.
   d. Heat shock the culture tubes for exactly 45 s at 42°C.
   e. Place tubes on ice 1–2 min.
   f. Add 500 μL pre-warmed S.O.C and recover 1 h at 37°C, shaking 200–250 rpm.
4. Plate dilutions of 1:10, 1:50 and 1:100 volume in total volume of 100 μL S.O.C and plate O/N at 37°C.
5. Screen colonies by colony PCR, or pick a colony to culture overnight, miniprep, and screen by restriction enzyme digest. Successfully cloned plasmids will be about 400 bp larger than the FP-tag backbone plasmid.
6. Sequence positive clones: Check that the Nb sequence is in frame with the florescent tag by sequencing from both ends (SP6 forward and SV40 reverse primers work well for this).

3.3 Generating Nb mRNA

It is crucial to keep rigorous glove and bench hygiene when handling templates and purified mRNAs, to avoid degradation by RNAses, which are ubiquitous in the environment.

1. Perform a *NotI* (New England Biolabs) digest of 3–5 μg of Nb plasmid template, per manufacturer instructions. Check digest completion by running an aliquot of the digest on an agarose gel.
2. Purify digested plasmid by DNA column preparation (Qiagen) or phenol-chloroform extraction and EtOH precipitation. Elute or resuspend the cut template in nuclease free water, usually <50 μL. A target concentration of >100 ng/μL is best for downstream synthesis reactions.

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3. Capped mRNA synthesis with SP6 mMESSAGE MACHINE (Ambion):

   a. 6 μL of cut template is the maximum amount for 20 μL synthesis reactions. The kit recommends 1 μg template, but synthesis occurs successfully with less; consider increasing the incubation time if significantly lower concentration template is used.

   b. In order to keep the temperature constant during the synthesis reaction, we recommend performing the reaction in PCR tubes incubated in a PCR machine with a hold temperature of 37°C.

   c. An incubation time from 2 to 4 h is sufficient for Nb mRNA synthesis.

   d. Finish with a 15-min rDNAse (Ambion) incubation at 37°C to remove the plasmid template. This is important to avoid the introduction of linearized plasmid into eggs during mRNA injection; linearized dsDNA is readily integrated into the genome.

   e. Precipitate the SP6 reaction with 30 μL 4 M LiCl in an overnight incubation at −20°C.

   f. Spin down RNA pellets at maximum speed at 4°C and wash two times with nuclease-free 70% EtOH. Resuspend in a small amount (<15 μL) nuclease free water to ensure high concentrations.

4. Aliquot 1 μL of fresh RNA into a 1:10 dilution with nuclease-free water to measure RNA concentration by Nanodrop or Qubit.

   a. Check the remaining aliquot on an agarose or RNA formaldehyde gel for intact bands; very smeary bands indicate degraded or poor mRNA quality.

5. Aliquot and store long-term RNA stocks at −80°C as soon as possible.

   a. Short-term working stock aliquots can be diluted to 100–500 ng/μL and stored at −20°C for 1–2 months before degradation issues crop up. Avoid repeated freeze-thaw cycles if possible.

   b. Periodic RNA gel checks are recommended to ensure fidelity of the working stock mRNA.

4 Titration and microinjection of Nb mRNA for live imaging and manipulation of target proteins in sea urchin embryo

The true power of nanobodies comes from their ability to be genetically encoded or ubiquitously expressed *in vivo*, depending on the transgenic or mRNA expression approach used. As a first pass when using the mRNA method in sea urchin embryos, especially for endogenous targets, we recommend co-injecting with proper controls. Co-injection with mRNA encoding the target antigen as a fusion protein with different spectral properties as the Nb is recommended to validate Nb specificity. For example, in order to validate the activity of a GFP-minimizing Nb (GBP4, Kirchhofer et al., 2010), we co-injected the

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Nb:GBP4^{mCherry} with ABCB1a^{GFP} or ABCB1a^{YFP} mRNA to co-localize GFP and Nb signals at the apical membrane (Fig. 2). Using this approach, we found that the GBP4 Nb also binds to YFP, but only quenches GFP fluorescence significantly.

Constitutive expression of tagged Nbs using the mRNA delivery method can result in a background of diffusely distributed fluorescence, if the mRNA injection concentration is too high. Thus, it is critical to titrate mRNA injection solutions such that excess, unbound chromobodies are minimized. In the sea urchin embryo, we have found that the chromobodies are best expressed at concentrations as low as 2–5 ng/μL mRNA, compared to 50–500 ng/μL mRNA of the target GFP fusion proteins. Multiple injection concentrations should be tried on at least three different mate pairs during optimization phases of Nb localization.

S. purpuratus microinjection protocols are provided in greater detail elsewhere (this volume; Rast, 2000). As a general overview, eggs are rowed onto filtered seawater (FSW)-filled petri dishes that are pre-coated with a 0.25% protamine sulfate (PS) solution. The PS coat enables the negatively charged eggs to adhere to the positively-charged plate. Rowed eggs are fertilized and immediately injected with a specialized microinjection needle. For embryos that will require long-term imaging, eggs should be rowed and injected directly on a PS-coated coverslip-bottom Delta-T or Mattek dishes. When outfitted with the correct cooling stages and high-speed laser scanners, *S. purpuratus* embryos can be maintained at 12–15°C and imaged by time-lapse microscopy for several hours without adverse effects or phototoxicity.

One advantage to injecting directly on glass-bottom plates is that the experimenter can inject every other embryo to ensure side-by-side comparison of Nb-injected to control wildtype embryos during imaging. With practice, one can inject one "round" of eggs with one mRNA concentration, and inject a second mRNA concentration (in a new needle) into the offset eggs in the second "round." In this case, it is better to row a conservative number of eggs, which will ensure that all eggs will be injected within the timeframe of the hardening fertilization envelope (<15 min). This approach only works for imaging time points prior to hatching, as the embryos will no longer stick to the plate once they have hatched from their envelopes (~18 hpf in *S. purpuratus*). Overall, we find this "50:50" dual injection approach particularly useful for streamlining the screening of Nb titrations and optimizing the imaging parameters to reduce background and noise.

4.1 Delivery of Nb mRNA by microinjection

1. Preparation of mRNA injection solutions, plates, and needles:

   a. Prepare injection solutions (total volume 3–5 μL) of mRNA in nuclease-free water. Keep on ice until ready to load the injection needle.

   b. Prepare a few different concentrations to optimize signal-to-noise ratio of localized Nbs (e.g., 1, 5, 10, 50 and 100 ng/μL to start).

   c. Score injection plates with a small scrape to provide a rigid surface for the needle to be broken open on.

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2. **2.** Preparation of eggs and injection plates for microinjection:

   a. Make a FSW-PABA (1–4 mM) solution to incubate eggs in rows for injection; the PABA softens the fertilization envelope to facilitate injection.

   b. Keep a FSW-ampicillin (50–100 μg/mL) solution on hand to replace the FSW-PABA post-injection.

   c. Spawn gametes. Collect sperm in a 0.5 mL Eppendorf tube and keep on ice. Keep eggs in a beaker of FSW and wash three times in FSW by letting them settle by gravity. Keep eggs as a monolayer to prevent hypoxia.

   For *S. purpuratus*, the following steps are best performed on a chill plate kept at 12–15°C:

   d. Fill several small watch glasses with FSW. Gentle swirling of watch glasses enables the quick pooling of eggs to facilitate transfer from one FSW wash to the next after dejellying.

   e. To remove the egg jelly-coat, place eggs into Acidic FSW (pH 4.8) by gently pipetting an aliquot into a watch glass filled with Acid-FSW. Let settle and swirl gently, until the eggs form a distinct spiral pattern; this often takes less than a minute or two. Once ready, immediately transfer pooled eggs back into normal FSW and proceed with three FSW washes. Alternatively, washed eggs can be sieved through a 120 μM Nitex mesh filter several times, followed by additional FSW washes into watch glasses.

3. Rowing and microinjection:

   a. Using a mouth pipet and a glass Pasteur pipet that has been drawn out to a diameter slightly larger than eggs (~80 μM in *S. purpuratus*), collect about 100 eggs at a time, and row eggs out in 2 or 3 single files onto the injection dish. Angle the tip of the pipet at a 45° angle and gently release eggs via mouth pipet, while gently tracing a line with the rowing filament end of the pipet.

   b. Load the microinjection needle with mRNA, position into the plate, and gently break the tip by jutting against the score mark.

   c. Fertilize eggs and commence microinjection as soon as fertilization envelopes have fully formed. Ensure that the injection solution looks like a small cloud entering the egg, but does not encompass more than ½ the egg volume. It is crucial to maintain consistent injection volumes for mRNA overexpression experiments (no >5–10% of total egg volume; 2–10 pL is a safe range for *S. purpuratus*).

   d. Immediately following injection, remove as much of the FSW as possible without exposing the eggs to surface tension or air.

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e. Replace with FSW-Amp to prevent bacterial growth on the leftover sperm stuck to the plate.

f. Incubate embryos at 12–15°C until imaging is required. It is good practice to keep injection dishes stored within a larger petri dish that also contains a moistened Kim-Wipe to provide some humidity.

4. Blastulae transfers:

   a. Unless you need to image early blastulae, transfer embryos to a new, uncoated petri dish containing fresh seawater. Embryos are best picked up and transferred by gentle mouth pipetting at pre-hatching stages (14–18 hpf).

   b. All embryos should be transferred or imaged within the first 24 hpf to avoid overgrowth of bacteria.

4.2 Imaging Nbs in early echinoderm embryos

General considerations and techniques for imaging echinoderm embryos with fluorescent confocal laser scanning microscopy (CLSM) are described in this volume. In our hands, single FP-tagged Nbs produce enough localized signal to be captured by more sensitive confocal detectors, such as the GaAsP and HyD detectors. We use a Leica SP8 CLSM equipped with HyD detectors, and can image physiological levels of chromobodies and the more abundant transporter proteins. Double or triple FP-tagged Nbs further enhance signal-to-noise ratio for illuminating physiologically relevant levels of target antigen. Multiple FP tags may prove mandatory to effectively image the Nb affinity reaction when using traditional photomultiplier tube equipped confocal microscopes.

1. Mounting embryos:

   a. Positive embryos can be screened and picked out of plates by using a stereoscope equipped with epifluorescence. Those injected with sufficient Nb or control fusion-FPs such as ABCB1a^{GFP} should weakly fluoresce when observing by eye pieces.

   b. For fast imaging, demobilize embryos by aliquoting several onto a 1% PS coated coverslip, with the coated side facing up and double-sided tape or plasticine feet added to the corners. Mouth pipetting enables the most control here; a plastic Pasteur pipette can be used for more dense cultures. The goal is to get as many embryos in the imaging frame as possible. Gently place a glass slide on top of the aliquoted embryos and adjust as necessary.

   c. For time-lapse or longer term imaging, immobilize embryos onto coated Mattek dishes. Image with a stage adaptor fitted with a cooling device or chamber to maintain culture temperatures.

   d. If injection was performed on Mattek or Delta-T dishes, proceed directly to imaging using an inverted objective microscope.

2. CLSM parameters for data collection:

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a. Image embryos by CLSM. On our Leica SP8 we use a 40× water-immersion objective with a 1.1-numerical aperture. Accompanied by <7× zoom levels, this provides adequate resolution to resolve micrometer scale structures without sacrificing signal brightness.

b. We prefer to maintain the pinhole at 1 Airy unit. Care should be taken to maintain identical detector gain and offset settings during each optimization experiment. Laser power should be monitored to ensure reproducibility between experimental batches.

c. If co-localizing the Nb with another FP, such as GFP (see Fig. 2), use appropriate filters to reduce cross-talk between channels. Sequential scanning setups will generally provide the least crosstalk if set up correctly.

d. Image embryos at 600–1000 hz, typically <2 μs per voxel. Full blastulae can be imaged in 1-μM thick z-sections for a total of ~70–90 slices.

e. Resonant scanning (at 8000 hz and higher) can be effective in reducing photobleaching while maintaining adequate spatial resolution.

3. Image processing and quantification:

a. Images are processed with ImageJ (NIH) or open source FIJI (Schindelin et al., 2012).

b. To quantify GFP fluorescence in the presence of the minimizing Nb:GBP4, measure the average GFP signal intensity by using the ROI manager. Draw a ROI along a GFP-positive area (e.g., apical microvilli in Fig. 2) within an equatorial z-slice, and the calculate average fluorescence value standardized to area. Compare to controls that were imaged with the same settings.

Note: For sharper image quality of apical membranes or vesicles, images can be smoothed by replacing each pixel with the average of its 3×3 neighbors using the Smooth tool in FIJI. Additional imaging and imaging processing details can be found in (Shipp et al. (2019).

5 Conclusions and future directions

Nanobodies are tools that can allow visualization of or perturbation of proteins in a spatially and temporally accurate manner *in vivo*. This technology perfectly leverages the core benefits of the sea urchin as a developmental model, namely the abundance, clarity and synchrony of the embryos. The example here shows the ease and high fidelity with which a GBP chromobody works in the echinoderm embryo, by utilizing simple mRNA overexpression methods. Endogenous targets will soon be visualized in similar fashion. It is also feasible to genetically encode GBPs, or any Nb for that matter, under the control of cell or tissue-specific promoters, through the mosaic integration of linearized plasmid or BAC-based reporter transgenes (Barsi, Tu, Calestani, & Davidson, 2015; Rast, 2000). When coupled with already available chromobodies targeting actin, vimentin, and cell-cycle

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antigens, the expression of cell-specific Nbs will enable researchers to illuminate cell division, migration, and EMT processes at an unprecedented level in F_0 embryos (Table 2). Adopting inhibitor Nbs to the fold opens the door for targeted, cell-specific protein perturbations to key signaling pathways such as Wnt/beta-catenin (Newnham et al., 2015) or even EMT events (Maier et al., 2015) during development.

In the context of studying gene regulatory networks (GRNs) Nbs can be harnessed for imaging TF translocation events, either through titrated overexpression of TF-specific chromobodies, or by adopting the GFP-trap approach of LlamaTags (Bothma et al., 2018). For the latter, mRNA overexpression could be used introduce an early ubiquitous GFP protein source into the embryo. Co-injection of TF-GBP fusion Nbs (LlamaTags) would illuminate TF cycling through the nuclearization of the GFP bait during development. Overexpressed (mRNA) or genetically encoded epitope-tagged Nbs (mosaic or CRISPR-mediated integration) could also be adopted for cheap pull-down assays of DNA-protein or protein-protein interactions. Notably, in model systems with stable GFP lines, the fusion of GBP Nbs to nuclease-dead Cas9 or fluorescent scaffolding proteins allows for the programming of chromatin remodeling events at specific loci, based on the recruitment of GFP-tagged co-factors and enzymes (Anton & Bultmann, 2017; Tang et al., 2013). These types of approaches complement efforts to generate genetically enabled lines of urchins.

The echinoderm research community could benefit immensely from the generation and sharing of Nanobody resources for commonly studied sea-urchin antigens. These include, but are certainly not limited to, transcription factors, primary and secondary mesenchyme cell markers, and signaling or membrane proteins (e.g., GCPRs, Delta/Notch, ABC transporters). In conclusion, bringing nanobodies into the Echinoderm toolbox will be transformative for understanding the constellation of protein-protein interactions, cytoskeletal re-organizations, signaling pathways, and gene-regulatory mechanisms that drive development of the early embryo. Nanobody technology could also be utilized to probe and perturb cellular responses to environmental or immune challenges in the embryo and larva. Complete integration of Nb technology in echinoderms will ensure that the only limit to experimental capabilities is not the animal model at hand, but the creativity of the researcher.

Appendix:

Resources for Nb derivation and fusion protein cloning

Animal nanobody production

1. Abcore (alpaca): https://discover.abcore.com/nanobodies/
2. ProSci (llama): https://www.prosci-inc.com/custom-antibody-services/single-domain-antibodies/
3. Creative Biolabs (llama and shark): https://www.creative-biolabs.com/Nanobody-Single-Domain-Antibody.html
4. Ablynx: http://www.ablynx.com/technology-innovation/nanobodies-competitive-features/

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Naïve or synthetic Nb libraries

1. Creative Biolabs: https://www.creative-biolabs.com/Premade-single-domain-antibody-library.html
   - CaV_HHL-1: Camel Naïve Single Domain Antibody Library
   - CaV_HHL-2: Camel Naïve Single Domain Antibody Library
   - $LlaV_HHL$-1: Llama Naïve Single Domain Antibody Library
   - Humanized camel libraries are available as well
2. Kruse Lab: First freely available synthetic library and related protocols (McMahon et al., 2018)

Nb databases and plasmid resources

1. *iCAN (Institute Collection and Analysis of Nanobody):* http://ican.ils.seu.edu.cn/Home/Index/can (Zuo et al., 2017)
   - Over 100,000 freely accessible sequences as of August 2018
   - Searchable though keywords (i.e., GFP, actin)
   - Video tutorial for searching and adding to the database
   - Downloadable search results include
     - Available DNA and protein sequences
     - Related publications (PubMed ID)
     - Source organism and experimental methods of screening
     - Function (i.e., crystallization or imaging)
     - Patent information
2. Addgene Plasmid Sources:
   - Hamdoun Lab pCS2 + 8 starter pack for cloning fusion proteins (Gökirmak et al., 2012)
   - Other Nbs: Over 40 plasmids for different antigens, expression systems, and applications are available on Addgene as of July 2018
3. Protein Databases:
   - EMBL-EBI: https://www.ebi.ac.uk
     - Backtranseq tool to generate sea urchin codon-optimized primary sequence (choose *S. purpuratus*): https://www.ebi.ac.uk/Tools/st/emboss_backtranseq/
   - Protein Data Bank: https://www.rcsb.org
4. Anti-Actin, Vimentin, and other V_{HH} plasmids for purchase: https://www.chromotek.com/products/chromobodies/actin-chromobodyr/

Cloning tools

1. *InFusion online tools:* https://www.takarabio.com/learning-centers/cloning/in-fusion-cloning-tools
    - Free web-based resources for designing cloning projects
    - Design primers
    - Molar ratio calculator
    - Final sequence predictor
2. *IDT Geneblocks*: https://www.idtdna.com/pages/products/genes-and-gene-fragments/gblocks-gene-fragments
    - Synthesize gene blocks of Nb of interest
    - Fast but expensive
3. *SnapGene Viewer*: https://www.snapgene.com
    - OpenSource: Versatile software to import or build, view, and edit plasmid sequences
    - SnapGene License: Additional cloning and alignment features available

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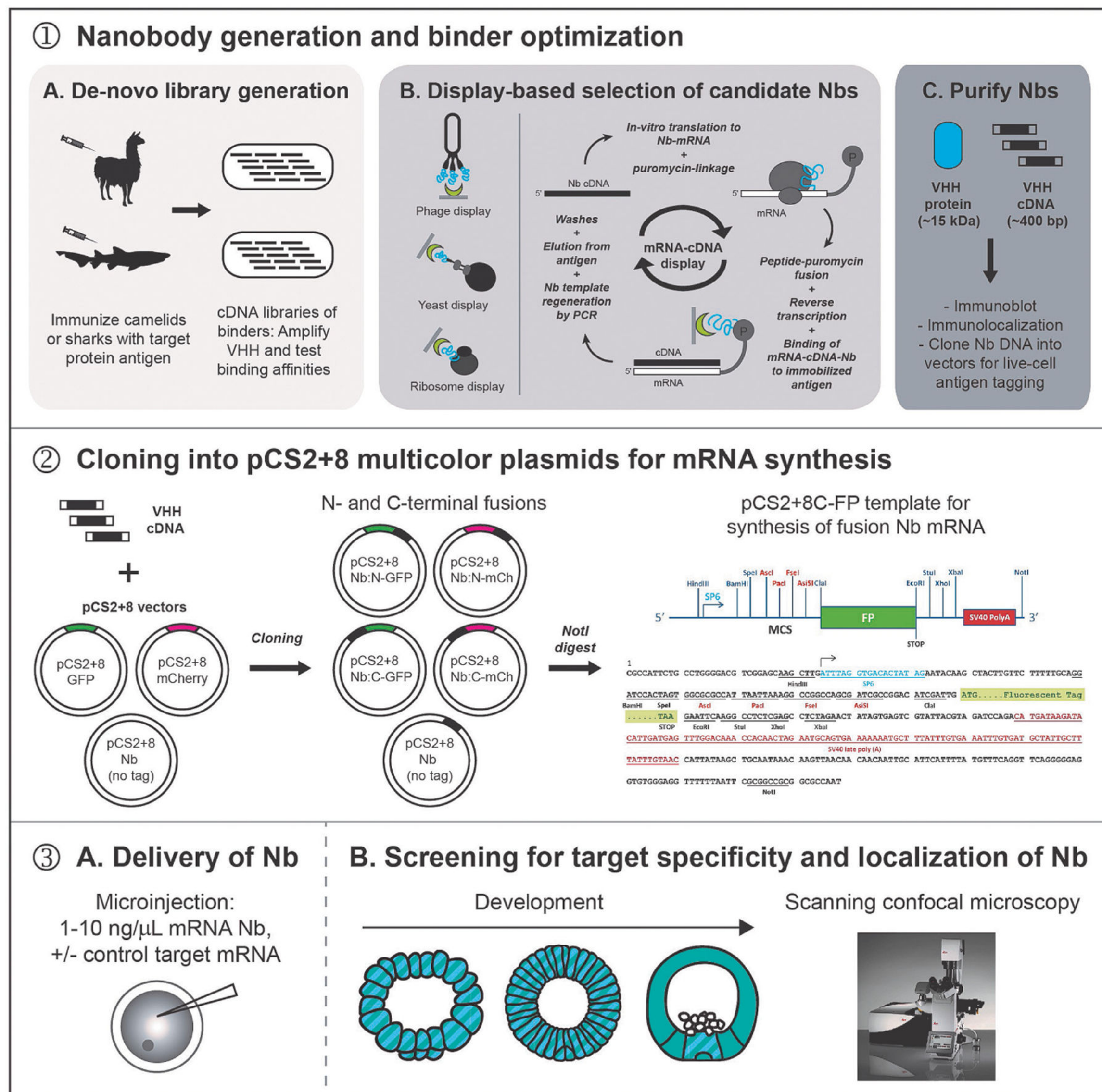

FIG. 1.

Overview of the generation and application of nanobodies in the sea urchin embryo. Three major steps are required to generate and optimize intracellular nanobodies (Nb) for live-cell imaging (LCAT) (corresponding to Sections 2–4 of the main text). The overall procedure involves (1) the selection of Nb candidates from immune, naïve, or synthetic cDNA libraries, (2) cloning of Nb sequences into vectors that generate Nb-fluorescent protein fusions (chromobodies), and (3) for the sea urchin system, zygotic microinjection of chromobody mRNA for LCAT of Nb protein in embryos. (1) Nanobody generation and binder optimization. Nanobodies can be generated from a variety of sources (see Appendix). cDNA libraries from immunized camelids or sharks (A) are screened for target specificity against immobilized antigen. Alternatively, cDNAs from naïve or synthetic libraries can be screened. Candidate binders are further optimized by rounds of phage-display, ribosome display, or mRNA/cDNA display. In mRNA/cDNA display (B), puromycin ligation

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technology permits the fusion of candidate binder cDNA with cognate mRNA and peptide. An mRNA template (white box) is covalently attached to puromycin. After *in vitro* peptide synthesis (light blue), puromycin linkage forms a covalent mRNA-protein fusion. Reverse transcription re-generates the cDNA template. The mRNA/cDNA-Nb complex is exposed to the antigen of interest (Ag, green crescent) immobilized on an affinity matrix. The matrix is washed, and the mRNA/cDNA-Nb complex is eluted. Ag-specific Nb sequences are retrieved by PCR on the eluted fraction. Error-prone PCR methods can be used at this step to generate mutated, potentially higher affinity cDNAs. (C) Following several rounds of recombination and binder pre-validation by immunoblot, ELISA or FACS, candidate Nb cDNAs are sub-cloned into vectors for large-scale protein production in *E. coli* or yeast. Proteins can be utilized in standard immunochemistry methods. Nb plasmids provide a template for downstream applications, such as the generation of Nb fusion proteins. (2) Cloning and synthesis of tagged fusion Nbs. Chromobodies for LCAT can be localized in sea urchin embryos by global mRNA overexpression (ubiquitous), or by linking *in vivo* Nb production with reporter-based expression (tissue-specific). The generation and titration of Nb mRNAs for ubiquitous Nb expression are the focus of this chapter. The pCS2 + 8 plasmid variants are designed for cloning and systematic screening of fusion proteins in the sea urchin embryo (Gökirmak et al., 2012). Nb sequences are sub-cloned into vectors encoding N- or C-terminal fusion proteins; the C-terminal fusion template is the tag of choice for most Nbs. mRNA is synthesized from linearized template using standard SP6 polymerase kits. (3) Microinjection, titration, screening and visualization of Nbs in live embryos. The expression of recombinant proteins using microinjected exogenous mRNAs is a routine technique to study protein localization in the sea urchin. (A) To adapt this method for LCAT, freshly fertilized eggs are injected with 1–10 ng/μL of Nb-mRNA. To validate Nb-antigen binding, mRNAs encoding the target protein tagged with fluorescent proteins of opposite spectral properties (e.g., $Nb^{mCherry}$ vs $TargetProtein^{GFP}$) are co-injected. Scanning confocal microscopy with highly sensitive photon detectors enables live-cell imaging and time-lapse analysis of Nb localization. The ease of microinjection and imaging in the sea urchin embryo allows for high-throughput titrations of Nb candidates to characterize endogenous or tag-specific (e.g., GFP) binders.

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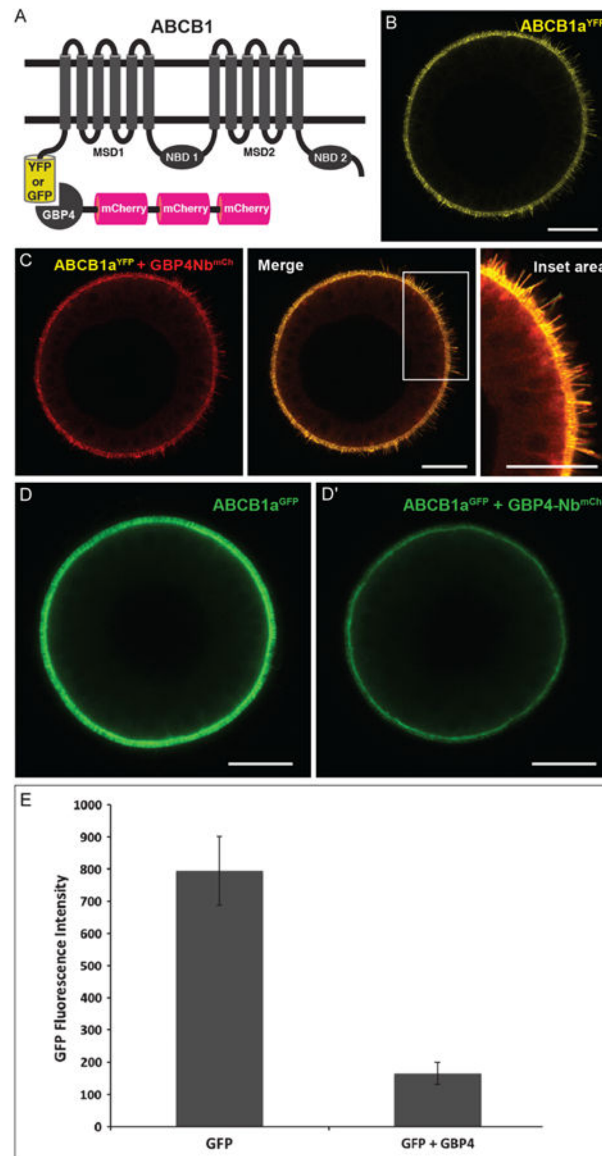

FIG. 2.

Live-cell antigen tagging and activity of a GFP and YFP-binding nanobody in the sea urchin embryo. The sequence for a GFP-minimizing nanobody (GBP4; Kirchhofer et al., 2010) was codon-optimized for *S. purpuratus* and cloned into a triple mCherry-tagging PCS2 + 8 expression vector to generate a C-terminal fusion nanobody (Nb). GBP4-Nb^{mCherry} mRNA was co-injected with mRNAs encoding a target GFP- or YFP-tagged ABC transporter membrane protein, ABCB1a. (A) Nb targeting scheme. ABCB1a$^{GFP/YFP}$ protein localizes to the apical membrane (Gökirmak et al., 2012), where it is targeted by the anti-GFP Nb. (B and C) GBP4-Nb^{mCherry} binds to a YFP target. Blastulae were imaged for ABCB1a^{YFP} fluorescence (yellow, B) and Nb signal (red, C) using scanning confocal microscopy. The GBP4-Nb^{mCherry} co-localizes with YFP with low background localization and no effect on YFP intensity. (D and E) Nanobody-mediated modulation of GFP fluorescence. Eggs were injected with ABCB1a^{GFP} and GBP4-Nb^{mCherry} mRNAs alone or in tandem. Blastulae were

imaged and quantified for GFP fluorescence using confocal laser scanning microscopy with HyD detectors. Single ABCB1a^{GFP} injected embryos (D) present typical GFP localization and intensity, whereas embryos co-injected with the GBP4-Nb^{mCherry} (D′) exhibit a fivefold reduction of GFP fluorescence (E). The average GFP signal intensity in epithelia from $n > 3$ embryos, sampled from three independent mate pairs, was quantified using FIJI imaging software. Scale bars, 20 μM. MSD, membrane spanning domain. NBD, nucleotide binding domain. DIC, differential interference contrast.

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Table 1

Applications of genetically encoded Nbs in developmental biology model systems.

Nb target	Design features	Applications	References	Model system
Tag targets				
GFP, RFP, or YFP	FP-enhancing or FP-minimizing	Super resolution microscopy; nuclearization assays of tagged estrogen receptors	Rothbauer et al. (2006) and Kirchhofer et al. (2010)	Mammalian cell lines
GFP	deGrad-FP: GBP fusion with E3 ligase	Protein knockdown; GFP-tagged proteins are targeted to the proteosome	Caussinus, Kanca, and Affolter (2012) and Shin et al. (2015)	*Drosophila*; mammalian cell lines; zebrafish
GFP	Morphotrap: GBPs linked to extracellular surface	Immobilize secreted GFP-tagged proteins on the surface of producing cells or surrounding tissue	Harmansa, Hamaratoglu, Affolter, and Caussinus (2015)	*Drosophila*
GFP	GrabFPs: GBP linked to cell compartments	GBP-based traps positioned along the apical-basal axis of epithelial cells; relocalize GFP-tagged proteins	Harmansa, Alborelli, Bieli, Caussinus, and Affolter (2017)	*Drosophila*
GFP	T-DDOG scaffolding proteins (nuclear)	GBP-mediated protein scaffolding directs transcriptional activity in GFP-expressing cells	Tang et al. (2013)	Mammalian cell lines; mouse; zebrafish
GFP	dCas9-GBP fusion Nb	Recruitment of GFP-tagged chromatin remodeling enzymes at specific loci	Anton and Bultmann (2017)	Mammalian cell culture
GFP	GBP Nb-TF fusion (LlamaTags)	TF-GBP fusions are used as bait for maternally derived GFP to visualize cycling of TFs	Bothma, Norstad, Alamos, and Garcia (2018)	*Drosophila*
GFP	GFP-minimizing GBP	GBP minimizer chromobody proof-of-concept in sea urchin		Sea urchin
Endogenous targets				
Actin	F-actin specific chromobody (non-inhibitory)	Visualize detailed cytoskeletal remodeling during cell division; development migrations	Panza, Maier, Schmees, Rothbauer, and Söllner (2015)	Zebrafish
	F-actin capping protein CapG-specific (inhibitory)	Inhibit cellular migration and cytoskeletal rearrangements	Van Impe et al. (2013)	Mammalian cell culture
PCNA	PCNA specific chromobody (non-inhibitory)	Monitor the cell cycle progression through S, G2, and M phases	Panza et al. (2015)	Zebrafish
Transcription factors	Beta-Catenin (inhibitory)	Inhibition of β-catenin's function as a transcriptional co-activator	Newnham et al. (2015)	Mammalian cell culture
Vimentin	Vimentin-specific (non-inhibitory)	Visualize vimentin dynamics during EMT events and EMT perturbation	Maier, Traenkle, and Rothbauer (2015)	Mammalian cell culture

Abbreviations: GBP, *GFP-binding protein;* GFP, *green fluorescent protein;* RFP, *red fluorescent protein;* YFP, *yellow fluorescent protein;* FP, *fluorescent protein;* T-DDOG, *transcription device dependent on GFP;* EMT, *epithelial to mesenchymal transition.*

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Table 2

Methods of Nb delivery in sea urchin embryos.

Delivery method	Design features	Applications
mRNA overexpression (microinjection)	Capped mRNA is translated to protein	Global, ubiquitous intracellular expression of chromobodies or other Nb-fusions
Cell-specific expression (microinjection)	Mosaic integration of an exogenous Nb transgene from plasmid or BAC sources	Specific promoters drive Nb expression in a cell-type or tissue specific manner
CRISPR/Cas9 transgenesis (microinjection)	Genomic integration of Nb transgene or epitope tags at endogenous loci	Nb is expressed from endogenous locus