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A transgenic approach to live imaging of heparan sulfate modification patterns

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Summary

Heparan sulfate (HS) glycosaminoglycan chains contain highly modified HS domains that are separated by sections of sparse or no modification. HS domains are central to the role of HS in protein binding and mediating protein-protein interactions in the extracellular matrix. Since HS domains are not genetically encoded, they are impossible to visualize and study with conventional methods *in vivo*. Here we describe a transgenic approach using previously described single chain variable fragment (scFv) antibodies that bind HS *in vitro* and on tissue sections with different specificities. By engineering a secretion signal and a fluorescent protein to the scFvs and transgenically expressing these fluorescently tagged antibodies in *Caenorhabditis elegans*, we are able to directly visualize specific HS domains in live animals (1). The approach allows concomitant colabeling of multiple epitopes, the study of HS dynamics and, could lend itself to a genetic analysis of HS domain biosynthesis or to visualize other non-genetically encoded or posttranslational modifications.

Keywords

heparan sulfate; single chain variable fragment (scFv) antibody; *Caenorhabditis elegans*; live imaging; non-genetically encoded molecules

1. Introduction

Heparan sulfate (HS) is an unbranched polysaccharide of repeating disaccharide units composed of *N*-acetylglucosamine and glucuronic acid (2). During synthesis, the disaccharide repeats are modified by a set of type II transmembrane Golgi-resident enzymes which can remove acetyl groups, add or remove sulfate groups, and convert glucuronic acid into the stereoisomeric iduronic acid (Fig. 1a)(7, 22). The glycan chain is invariably attached via a characteristic tetrasaccharide linker to a serine on core proteins that include the extracellular membrane-attached syndecans and glypicans as well as secreted perlecan and

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collagen XVIII (2). Core proteins, together with their glycan chains, are referred to as heparan sulfate proteoglycans (HSPG)(2).

HSPGs have been shown to serve diverse functions in physiology and development (3, 5, 26). Many of these functions are mediated by specifically modified HS modification patterns interacting with a variety of molecules, including growth factors and receptors. At least some of these protein-glycan interactions are highly specific (such as the antithrombin III binding to a characteristic pentasaccharide) whereas other interactions may be less specific (18). Highly modified regions of the HS chains are interspersed with less modified or unmodified regions where the distance between modified domains is essential for function (31). The HS domains appear highly specific in their tissue and age distribution (35). However, without live imaging approaches it has been impossible to study the developmental dynamics of HS domains *in vivo*, or to pursue genetic approaches to understand HS biosynthesis on a mechanistic level. Single chain variable fragment (scFv) antibodies are synthetic antibodies based on human heavy and light chain variable regions that have been assembled *in vitro* (17, 24). By randomizing the complementarity determining region 3, vast phage display cDNA libraries of scFv antibodies have been created (17, 24). By panning these libraries with an epitope of interest, scFvs against essentially all types of epitopes have been identified, including glycosaminoglycans such as HS, which are notorious for displaying low antigenicity in traditional approaches (37). Using a number of different HS/heparin preparations, van Kuppevelt and colleagues identified at least 36 scFv antibodies that bind HS or heparin with varying specificities (6, 19, 32–34, 36, 37, 39). Some of these scFv antibodies have been characterized as binding to specifically modified heparan sulfate oligosaccharides *in vitro* (e.g. AO4B08 and HS4E4, (21)) but the majority has not been molecularly characterized in detail.

Caenorhabditis elegans is widely used in genetic and neuroscience research owing to its transparency, the ease of transgenic approaches and the possibility to utilize fluorescent proteins to analyze and characterize cells, organelles, and proteins in high resolution. The *C. elegans* genome encodes one of each class of the heparan sulfate modifying enzymes (5), making this nematode an excellent system to study the genetic underpinnings of heparan sulfate modifications during development and physiology. We have used *C. elegans* and transgenically expressed anti-heparan sulfate specific scFv antibodies to devise a system for the *in vivo* study of non-genetically encoded HS modification patterns (Fig. 2)(1).

2. Materials and Equipment

2.1 Nematode husbandry

1. Nematodes and the OP50 *E. coli* feeding strain can be obtained from the *Caenorhabditis elegans* Genetics Center at the University of Minnesota (www.cbs.umn.edu/cgc).
2. Nematode Growth Medium (NGM) agar plates for growing worms require 60 mm diameter petri plates (e.g. from Tritech Research, www.tritechresearch.com), a peristaltic pump or 50 mL serological pipettes and a pipetting ball.
3. A dissecting microscope with a transmitted light base (e.g. Zeiss Stemi 2000).

4. Platinum wire 99.95% platinum, 0.05% iridium 0.01 inches diameter wire (e.g. PT-9901, Tritech Research, www.tritechresearch.com), Glass Pasteur pipets, 1000 μ L pipette tips, hair (from eyelash, pet whisker, or paint brush), cyanoacrylate adhesive “superglue”.

2.2 Solutions and Media

1. M9 buffer solution: 3 g KH_2PO_4 , 6 g Na_2HPO_4 , 5 g NaCl, 1 mL 1 M MgSO_4 , H_2O to 1 liter. Sterilize the solution and store it at room temperature.
2. LB media: 10 g tryptone, 5 g yeast extract, 5 g NaCl, H_2O to 1 liter. Autoclave the solution and store it at 4°C.
3. 40 mM levamisole stock solution: 0.0963 g levamisole hydrochloride, 10 mL ddH_2O . The solution should be aliquoted and stored at -20°C .
4. 0.6 M 2,3-butanedione monoxime (BDM) stock solution: 0.121 g 2,3-BDM (B0753 Sigma-Aldrich), 2 mL M9 buffer solution. This solution should be prepared fresh each day.
5. 3% agarose for pads: 3 g agarose, 100 mL ddH_2O . Bring to a boil and keep on a hot plate.
6. LB agar plates:
 1. Agar solution: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar, H_2O to 1 liter. Autoclave the solution.
 2. Cool flask in 55°C water bath for 15 minutes.
 3. Add 1 mL of ampicillin (from stock solution of 100 mg/mL in ddH_2O) before pouring plates if needed.
 4. Pour approximately 20 mL of agar into 10 cm petri dishes using peristaltic pump or serological pipettes.
7. NGM (*Nematode Growth Medium*) plates:
 1. Agar solution: 17 g agar, 3 g NaCl, 2.5 g peptone, 975 mL ddH_2O . Combine in a 2-liter Erlenmeyer flask, cover with aluminum foil, and autoclave.
 2. 1 M KPO_4 buffer: 108.3 g KH_2PO_4 , 35.6 g K_2HPO_4 , ddH_2O to 1 liter.
 3. Cool flask in 55°C water bath for 15 minutes.
 4. Add 1 mL 1 M CaCl_2 , 1 mL 5 mg/mL cholesterol in ethanol, 1 mL 1 M MgSO_4 , and 25 mL 1 M KPO_4 buffer. Mix the solution.
8. Pour approximately 10 mL into 60 mm petri dishes using peristaltic pump or serological pipettes.

2.3 scFv constructs

1. Competent bacterial cells for molecular biology.

2. Site-directed mutagenesis kit (e.g. Agilent QuickChange II).
3. LB agar plates with ampicillin.
4. Restriction enzymes (depending on subcloning goals) and materials for standard molecular biology techniques as needed.

2.4 Microinjection and imaging

1. A standard set-up for microinjection of nematodes is required, including an inverted microscope (e.g. Zeiss Axio Observer), a needle holder (e.g. microINJECTOR Brass Straight-Arm Needle Holder MINJ-4 from Tritech Research, www.tritechresearch.com) combined with a micromanipulator (e.g. Three-Axis Oil Hydraulic Fine Micromanipulator MMO-203, Tritech Research, www.tritechresearch.com), compressed nitrogen gas tank and a pedal-operated valve for injection with pressurized nitrogen (e.g. microINJECTOR System MINJ-1 Tritech Research, www.tritechresearch.com).
2. Microscope slides (75 x 25 mm) and cover slips (60 x 24 mm for injection pads and 22 x 22 mm for imaging) are necessary for microinjection and imaging. Halocarbon Oil 700 (H8898 Sigma-Aldrich) is used to immerse and immobilize the worms on the slides.
3. Needles: Microinjection requires thin glass needles that can be made from thin-walled 1.0 mm outer diameter 0.75 mm inner diameter borosilicate glass capillaries with an internal glass filament (e.g. TW100F-4 World Precision Instruments). These glass capillaries are pulled into two needles using a needle puller such as a Narishige PC-10. The settings of the needle puller vary depending on the make and model as well as the filament age and have to be determined by trial and error. Generally, needles should taper quickly to a fine point. If the needle tapers too slowly or too fast the needle may bend too much to penetrate the cuticle of the worm or could puncture too large of a hole in the cuticle causing the worm to burst.
4. Agarose pads for injection: Prepare a 3% agarose solution in water by bringing 3 g agarose to a boil in 100 mL ddH₂O until completely dissolved. 3% agarose can be kept on a heat block for a day. Use a glass Pasteur pipette where the tip has been broken to create a wider opening and place around 100 µL of molten agarose onto a glass slide coverslip (50 x 22 mm). Place another coverslip on top at a 60° angle and lightly compress until the agarose is spread evenly and thinly. The slide can be left to cool while more slides are prepared. Cooled slides are separated and the coverslip with the agarose should be placed in an 80°C vacuum oven with the agarose facing up for one to four hours. Generally, mounted worms will stick more to a drier pad but they will also desiccate more quickly. Thus, depending on the environmental conditions (humidity etc.) the optimal drying time of agarose pads has to be determined experimentally, but 2 hrs are a good starting point.

3. Methods

3.1. Designing the scFv constructs for transgenic expression

1. The scFv antibodies contain a variable heavy and a variable light chain (Fig. 1b). For all antibodies in this set, the variable light chain sequences are identical (37). The heavy chain sequences fall into 13 families. Within a family, all members have identical heavy chain sequences except for the complementarity determining region 3 (CDR3) which is unique for each antibody within the family. Therefore, we initially synthesized all individual heavy chains as well as the common light chain *de novo* with codon usage optimized for *C. elegans*. Unique restriction sites (Fig. 1b, Note 1) allowed convenient assembly into a founding member of each class of scFvs that share a common variable heavy chain. Using these constructs as templates, we established all other scFv antibody constructs of this family by site-directed mutagenesis of the CDR3 sequence. For example, HS3A8 was derived from HS4C3 by mutagenesis using primers that replace the CDR3 of HS4C3 with the CDR 3 of HS3A8, because both scFvs share the heavy chain of family DP-38 (e.g. HS4C3 CDR 3: '... VYYCARGRRLKDWGQGT...' is altered to HS3A8 CDR 3 '... VYYCARGMRPRLWGQGT...')(1). Appropriate controls to demonstrate specificity of any observed staining have to be included (Note 2).
2. The scFv heavy and light chains need a secretion signal to target the scFvs to the extracellular space and allow labeling of extracellular matrix components. We have used the secretion signal of *sel-1* (14). Conceivably, other sequences could be fused to the scFvs instead for targeting to other cellular compartments if so desired, such as for example to the nucleus, the cytosol or the endoplasmic reticulum. To the C-terminus, we fused in frame a fluorescent protein (Fig. 1b, for a discussion of different fluorescent proteins see Note 3).
3. To control the level of secretion, an appropriate promoter for transgenic expression needs to be chosen (for a discussion of promoter choice see Note 4). The level and location of expression both need to be taken into account. Unlike in immunohistochemistry, where the level of antibody can be directly controlled, the level of antibody is mainly determined by the strength of the promoter used. The *unc-122* promoter (23) proved to be an excellent promoter in that it limits expression to the coelomocytes (a set of scavenger cells)(Fig. 1c) and has a moderate level of activity so that background fluorescence is kept low. If specific staining is detected, alternative promoters should be used to confirm results obtained with the coelomocyte-specific promoter, such as the *glo-1* promoter which drives expression in the intestine (16). Regardless of the promoter used, antibodies will be visible in vesicles of the coelomocytes which are known to uptake proteins from the pseudocoelom (i.e. the extracellular space)(Fig. 2a,b).
4. All of these components are combined into a single plasmid construct. Sequences for all existing HS specific scFv constructs are available on request.

3.2. Nematode husbandry

1. The method in its current form has been established using the nematode *Caenorhabditis elegans*, has been used in at least one related species (see Note 5). Worms are maintained using standard procedures and kept at 20°C (4).
2. Overnight cultures of the uracil auxotroph *Escherichia coli* strain OP50 grown in LB media are used as a food source. Generally, 500 µL of a bacterial overnight culture seeded on a 60 mm NGM plate produces a large bacterial lawn that covers approximately 75% of the agar surface. The bacterial lawn takes one or more days to dry depending on the age of the plates, humidity, etc.. Appropriately dried plates should neither contain remaining liquid culture nor show a cracked agar surface.
3. Worms are moved between plates using a ‘pick’ made from platinum wire fixed to a glass Pasteur pipette. The front of the ‘pick’ should be flattened to make a spade-like shape to aid in the movement of worms between plates. To move worms between plates and a microinjection slide, use a thin but resilient hair such as an animal whisker, human eyelash, or paint brush hair, fixed to a pipette tip with super glue. Cat whiskers can be cut at an angle so that the tip is flatter and can better scoop worms off the plates.

3.3. Transgenesis

1. Here, the “complex arrays” approach is discussed (see Note 6). Prepare the injection mixes for the generation of multi-copy arrays as an aqueous solution. Include 5 ng/µL linearized plasmid containing the scFv antibody construct, 5 ng/µL linearized dominant injection marker plasmid such a fluorescent marker for the cells of the gut, and digested genomic DNA from *C. elegans* as a carrier up to a final combined concentration of at least 100 ng/µL. Other concentrations of plasmids are possible but these concentrations are a good start.
2. Pull needles using needle puller and glass capillaries containing an internal filament. If the needle tapers too slowly or too quickly, the needle may bend too much to penetrate the cuticle of the worm or could make a hole in the cuticle, respectively causing the worm to burst.
3. Prepare agarose pads for injection.
4. Arrange all materials for microinjection: injection needles, injection pads, halocarbon oil, M9 buffer, a hair pick, and young, healthy and well-fed adult *C. elegans*.
5. Load injection needles using a micropipette with 1 µL of injection mix from the back end (let the capillary forces pull in the solution) and mount the loaded needle into a microinjector/needle holder.
6. Prepare the injection pad by placing a drop of halocarbon oil on the dried agarose pad.

7. Prior to mounting worms onto the dried agar pad, position the needle into view and check if the needle is open or if it needs to be broken. Use the edge of the oil drop on the dried agar pad to focus and align the needle in the field of view. Bring the tip of needle into the oil. Test the needle by trying to force out the aqueous injection mix into the oil. If the solution flows freely, there is no need to break the needle. If there is no flow, the needle tip needs to be broken. This can be accomplished by gently dragging the needle tip along the dried agar of the pad while holding the pedal down, i.e. applying gas pressure. Once the tip breaks, the injection mix will flow out driven by the flow of compressed nitrogen. Depending upon the needle, the gas pressure should be between 200 and 350 kPa. Be careful to not break the needle too far from the tip as a wide bore may damage the worms.
8. Worms should be injected as young, healthy and well-fed adults. These animals can be identified by the number of eggs inside the animal. A young adult will usually contain 6 to 12 embryos. Pick animals using the hair pick and transfer them into the oil drop on the agarose pad. Try to pick worms without bacteria attached (e.g. by picking worms from outside the bacterial lawn), because attached bacteria may create difficulties during injections (such as variable adhesion to the injection pad). Manipulate worms such that they are lined up in a convenient orientation where the distal arms of the gonad are visible in the field of view (at 200x magnification). Once the worm is in a good orientation, press the animals gently through the oil onto the agarose, where they should begin to stick due to desiccation. At this point, there is a limited amount of time (not more than 10 to 20 minutes) before the worms die due to progressive desiccation. Thus, work quickly and deliberately as time may be one of the determining factors in being successful with microinjections.
9. Once the worm(s) are mounted onto the agarose pad, move the cover glass to the microscope for microinjection. A skilled injector may be able to inject as much as 20 animals all lined up on the pad together; a novice should start with 2. Target the needle for microinjection to the distal arm of the gonad, which has a characteristic tubular appearance containing spherical germ cells with large nuclei. This can be clearly seen with differential interference microscopy (DIC). When the needle is correctly inserted in the distal arm, press the pedal that controls gas flow through the needle; there should be a noticeable flow of injection mix into the distal arm traveling towards the vulval opening. The increased pressure in the gonad sometimes results in the extrusion of an egg. If the needle is incorrectly positioned in the pseudocoelom, the flow will instead push the gut and gonad apart. For an extended discussion of microinjection of nematodes, see (8).
10. Once injected, recover the worms by rescuing them in a drop of M9 buffer which will free the worms from the dried agar pad. Then, use the hair pick or an aspirator to move worms from the pad back to a seeded NGM plate. Once the animals have recovered for an hour, move them to individual NGM plates to lay a brood.

11. After eggs are laid and grow up to the late larval or early adult stages, screen the progeny (F1 generation) for expression of the dominant injection marker that was used. Move these transgenic F1 animals to new individual plates and keep until their progeny (F2 generation) are old enough to screen for germline transmission of the transgene. Often, about 1 in 10 transgenic F1 animals produces transgenic progeny, i.e., result in animals with germ line transmission. The ratio between the total number of F1 animals and those that result in germline transmission depends upon the injector and the injection mix (complex array mixes sometimes have a higher rate of successful transmission). An F1 animal that produces consistent germline transmission is considered a clonal extrachromosomal transgenic line, which has to be maintained by picking transgenic animals.

3.4. Imaging transgenic animals

1. Prepare well-fed animals of the desired stage for imaging. Animals can be visualized at any stage but visualization may be easier at younger stages. For example, animals expressing antibodies under control of the *unc-122* promoter are best viewed during the early larval stages as the *unc-122* promoter is most active from late embryonic to mid-larval stages (for a discussion on promoter choice see Note 4).
2. Prepare anesthetics, 1.5 mL microcentrifuge tubes, imaging slides, and transgenic worms for imaging (for a discussion of anesthesia see Note 7). Wash worms off the plate into 1.5 mL microcentrifuge tubes using M9 buffer. Gently pellet the worms by centrifugation using a low setting (2000 rpm) for 2 minutes. Decrease the volume of the tube to 20 μ L and add 20 μ L of 16 mM levamisole. Then add 40 μ L of 0.6 M 2,3-BDM to the tube and gently mix. Allow worms to settle or pellet using a microcentrifuge at a low setting. Worms will collect at the bottom of the tube and be ready for mounting.
3. Prepare slides one or two at a time as worms anesthetized with this solution will start dying after 30 minutes. Slides for imaging should be freshly made with 3% agarose pads prepared in similar fashion as the pads for injection but on microscope slides and without drying. Transfer worms to a slide with an agar pad using a cut pipette tip so as to not damage the worms. Once the worms are on the slide, gently lay a cover glass onto the agar pad over the worms. Mount worms in an appropriate volume of liquid to avoid crushing them (too little liquid) or having them float too freely (too much liquid) (approximately 8 μ L for a 25 x 25 mm cover slip).
4. The fluorescence from the antibodies can be weak. Thus, limit exposure to light, particularly at excitatory wavelength, to avoid bleaching. Increase the camera gain as high as necessary to produce a usable image that limits exposure length and minimizes noise.

4. Notes

4.1. Modular nature of scFv constructs

The modular nature of the constructs allows convenient conversion into different constructs with different specificities. For example, if scFvs against other molecules of interest become available, such as other glycans, small molecules or against post-translationally modified molecules (e.g. a phosphorylated or glycanated protein of interest), it should be possible to merely exchange the variable heavy chain as needed. If the heavy chain backbone is available already, this may even be accomplished through simple site-directed mutagenesis.

4.2. Appropriate controls for transgenic scFv antibody approaches

Appropriate controls for transgenic scFv approaches are essential. First, one has to ascertain that the observed staining is specific for the scFv at hand and not the fluorescent protein. For example, in the case of anti-HS scFv antibodies, a control scFv antibody fusion (MPB49::GFP) was expressed under identical conditions which did not result in comparable staining (1). Second, using genetic methods it has to be tested whether the scFv antibody does indeed recognize the epitope of interest. For HS this has been accomplished in several instances by demonstrating dependence of staining on the presence of certain genes of the HS biosynthetic machinery (1).

4.3. Fluorescent tag choice

The fluorescent tag used for visualization is extremely important. The GFP version (which carries a S65T mutation) described here is good starting point. But for experiments that involve detection of multiple epitopes, antibodies fused to different fluorescently labeled proteins are required (Fig. 2c–d). In principle, the antibody constructs can contain any fluorescent marker, but usability is limited by the intensity of the fluorescent protein. The super folder GFP (28) is a bright alternative to EGFP. DsRed2, tagRFP, and mCherry work, but DsRed2 and mCherry may show some aggregation in the extracellular space. Nonetheless, double labeling experiments with different fluorophores are possible (Fig. 2c, d). Current CFP, YFP, and BFP fluorescent markers appear too weak to be used successfully with this system, but perhaps optimized variants for the extracellular space may prove usable in the future. Beyond fluorophore choice, multimerizing the super folder GFP (a dimer with a short linker sequence) resulted in a brighter signal and allowed better visualization of weaker or less common epitopes. Perhaps producing longer chains of fluorophores may mimic the signal-amplification property of secondary antibodies. Alternatively, non-fluorescently-tagged scFv antibodies could be paired with transgenically-expressed, fluorescently-labeled secondary antibodies to provide signal amplification.

4.4. Promoter choice

It is important to choose an appropriate promoter to drive expression. Keep in mind that the fluorescence of the cells that secrete the antibody may obscure staining on the surface of those cells. Choose cells that are not of interest or unlikely to have staining. Based on these considerations the use of the *unc-122* promoter (23) was an obvious choice and convenient for three reasons. First, it expresses the antibody at a level where it could be visualized

without being so high that fluorescently labeled scFv started aggregating in the extracellular space. Second, the coelomocytes are not absolutely fixed to the same position, but are at times found in slightly different positions in the pseudocoelom of *C. elegans* so that no location is always obscured by the source of the antibody. And third, the coelomocytes take up proteins in the pseudocoelom (9) and therefore would eventually contain the fluorescent antibody regardless of whether they produce it. Thus, instead of two sets of cells being 'unusable' (because of internal fluorescent signal), only one set is.

Promoters like the *glo-1* promoter (16) provide a slightly weaker level of expression than the coelomocytes and most structures visible with the *unc-122* promoter are also visible. Other promoters like the *myo-3* or *grd-10* promoters (15, 27) drive expression in muscle or the seam cells so strongly that specific and non-specific staining cannot be distinguished easily. Initial experiments with *C. elegans* heatshock promoters were not successful, though perhaps with alternative activation lengths or temperatures they might produce usable levels of expression. Chemically inducible systems (10, 29) may provide another alternative approach and may allow expression using stronger promoters (*myo-3* or *grd-10*) by tightly controlling expression before too much antibody is produced. Ideally, a combination that allows both spatial and temporal control of expression may produce the best results such as the recently developed Q-system (29, 38).

4.5. Applying this technique beyond *C. elegans*

While the described method uses *C. elegans*, this technique could also be applied to other organisms. To date, the technique has been applied to one other nematode species, *Caenorhabditis briggsae* (Attreed & Bülow, unpublished), but could possibly be applied to any nematode species for which transgenesis methods are available, or even non-nematode model organisms such as flies or fish. A possible concern is that the antibody-fusions are continuously produced and could build up in the extracellular space as an animal ages. One possible path around this is to use heat shock promoters or a chemically induced system (tamoxifen-Cre or quinic acid-Q System)(10, 29). Another possibility would be to choose a promoter with temporally limited expression. The coelomocytes in *C. elegans* absorb the antibody from the extracellular space and likely play a key role in keeping background levels of staining low by absorbing unbound antibody (13). Other species have similar cells or organs in charge of this process. Second, *C. elegans* is transparent throughout development. Other organisms, such as *Drosophila* and zebrafish, have more transparent stages early in development only. For organisms that are more opaque, far red fluorescent tags have been shown to work *in vivo* in adult mice (11) and could possibly be employed. Finally, most organs in *C. elegans* are exposed to the pseudocoelom; in other organisms, there is more tissue compartmentalization and a freely diffusing, fluorescently labeled antibody may have a more limited reach. Thus, the use of more region-specific expression of the antibodies may be required to see staining of, for example, specific areas of the nervous system.

4.6. Discussion of transgenic approaches

Several approaches exist to create transgenic animals in *C. elegans* (8), including the generation of multi-copy extrachromosomal arrays by microinjection (25), methods for single copy insertion (12) and bombardment (30). All experiments to date were performed

using multi-copy arrays, including both “standard arrays” and “complex arrays”. For “standard arrays” injection mixes containing circular plasmids where 25 ng/μL should be used for the antibody plasmid and 25–50 ng/μL for the marker plasmid. The rest of the mix, up to a total DNA concentration of 100 ng/μL, should be comprised of generic vector plasmids such as pBluescript or pUC19.

“Complex arrays” mixes only contain linearized DNA. DNA can be linearized by any restriction enzyme that cuts plasmids once in the backbone and cuts genomic DNA approximately every 1 to 2 kilobases. Mixes may contain as little as 5 ng/μL of the linearized antibody plasmid along with 5 ng/μL of a dominant injection marker plasmid. The rest of the mix is comprised of digested genomic DNA from *C. elegans* or *E. coli* to a total DNA concentration of 100 ng/μL. The complex array injection mixes have the added benefit of better transmission between generations and better expression. Alternative methods such as single copy insertion (12) and bombardment (30) could also work, although in the case of single copy insertion, the expression may be too weak under a promoter like the *unc-122* promoter. In this case, perhaps a stronger promoter such as the *myo-3* promoter could be used to increase expression. In summary, a variety of transgenic approaches exist and the optimal approach may have to be determined experimentally with “complex arrays” being a convenient starting point.

4.7. Anesthesia

The choice of anesthetic is important. Poorly anesthetized worms may move or twitch during the exposure under the microscope making imaging of weak signals particularly difficult. *C. elegans* tend to twitch when exposed to blue light, which exacerbates this problem. This problem, however, can be managed by using combinations of anesthetics. Sodium azide is a common anesthetic, but tends to weaken fluorescence, particularly fluorescence of extracellular molecules. Levamisole proved to be a better choice because it failed to weaken the fluorescence, though it sometimes lead to morphological changes and “bubbles” near the nerve ring. Since levamisole by itself did not completely inhibit muscle function, and muscles in the pharynx often still twitched, a combination of anesthetics turned out to be the best approach. When levamisole was combined with 2,3-BDM, the worms became completely immobilized, but lived only between 30 and 60 minutes on the slide before dying.

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References

1. Attreed M, Desbois M, van Kuppevelt TH, Bulow HE. Direct visualization of specifically modified extracellular glycans in living animals. *Nat Methods*. 2012; 9:477–479. [PubMed: 22466794]
2. Bernfield M, Götte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem*. 1999; 68:729–777. [PubMed: 10872465]
3. Bishop JR, Schuksz M, Esko JD. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature*. 2007; 446:1030–1037. [PubMed: 17460664]

4. Brenner S. The genetics of *Caenorhabditis elegans*. *Genetics*. 1974; 77:71–94. [PubMed: 4366476]
5. Bülow HE, Hobert O. The Molecular Diversity of Glycosaminoglycans Shapes Animal Development. *Ann Rev Cell Dev Biol*. 2006; 22:375–407. [PubMed: 16805665]
6. Dennissen MA, Jenniskens GJ, Pieffers M, Versteeg EM, Petitou M, Veerkamp JH, van Kuppevelt TH. Large, tissue-regulated domain diversity of heparan sulfates demonstrated by phage display antibodies. *J Biol Chem*. 2002; 277:10982–10986. [PubMed: 11790764]
7. Esko JD, Selleck SB. Order out of chaos: assembly of ligand binding sites in heparan sulfate. *Annu Rev Biochem*. 2002; 71:435–471. [PubMed: 12045103]
8. Evans, T. Transformation and microinjection. *WormBook*; 2006.
9. Fares H, Greenwald I. Genetic analysis of endocytosis in *Caenorhabditis elegans*: coelomocyte uptake defective mutants. *Genetics*. 2001; 159:133–145. [PubMed: 11560892]
10. Feil R, Wagner J, Metzger D, Chambon P. Regulation of Cre recombinase activity by mutated estrogen receptor ligand-binding domains. *Biochem Biophys Res Commun*. 1997; 237:752–757. [PubMed: 9299439]
11. Filonov GS, Piatkevich KD, Ting LM, Zhang J, Kim K, Verkhusha VV. Bright and stable near-infrared fluorescent protein for in vivo imaging. *Nature biotechnology*. 2011; 29:757–761.
12. Frokjaer-Jensen C, Davis MW, Hopkins CE, Newman BJ, Thummel JM, Olesen SP, Grunnet M, Jorgensen EM. Single-copy insertion of transgenes in *Caenorhabditis elegans*. *Nat Genet*. 2008; 40:1375–1383. [PubMed: 18953339]
13. Gottschalk A, Schafer WR. Visualization of integral and peripheral cell surface proteins in live *Caenorhabditis elegans*. *J Neurosci Methods*. 2006; 154:68–79. [PubMed: 16466809]
14. Grant B, Greenwald I. The *Caenorhabditis elegans* sel-1 gene, a negative regulator of lin-12 and glp-1, encodes a predicted extracellular protein. *Genetics*. 1996; 143:237–247. [PubMed: 8722778]
15. Hao L, Johnsen R, Lauter G, Baillie D, Burglin TR. Comprehensive analysis of gene expression patterns of hedgehog-related genes. *BMC Genomics*. 2006; 7:280. [PubMed: 17076889]
16. Hermann GJ, Schroeder LK, Hieb CA, Kershner AM, Rabbitts BM, Fonarev P, Grant BD, Priess JR. Genetic analysis of lysosomal trafficking in *Caenorhabditis elegans*. *Mol Biol Cell*. 2005; 16:3273–3288. [PubMed: 15843430]
17. Hoogenboom HR, Winter G. By-passing immunisation. Human antibodies from synthetic repertoires of germline VH gene segments rearranged in vitro. *Journal of molecular biology*. 1992; 227:381–388. [PubMed: 1404359]
18. Jakobsson L, Kreuger J, Holmborn K, Lundin L, Eriksson I, Kjellen L, Claesson-Welsh L. Heparan sulfate in trans potentiates VEGFR-mediated angiogenesis. *Developmental cell*. 2006; 10:625–634. [PubMed: 16678777]
19. Jenniskens GJ, Oosterhof A, Brandwijk R, Veerkamp JH, van Kuppevelt TH. Heparan sulfate heterogeneity in skeletal muscle basal lamina: demonstration by phage display-derived antibodies. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2000; 20:4099–4111. [PubMed: 10818145]
20. Jorgensen EM, Hartwig E, Schuske K, Nonet ML, Jin Y, Horvitz HR. Defective recycling of synaptic vesicles in synaptotagmin mutants of *Caenorhabditis elegans*. *Nature*. 1995; 378:196–199. [PubMed: 7477324]
21. Kurup S, Wijnhoven TJ, Jenniskens GJ, Kimata K, Habuchi H, Li JP, Lindahl U, van Kuppevelt TH, Spillmann D. Characterization of anti-heparan sulfate phage display antibodies AO4B08 and HS4E4. *J Biol Chem*. 2007; 282:21032–21042. [PubMed: 17517889]
22. Lindahl U, Kusche-Gullberg M, Kjellen L. Regulated diversity of heparan sulfate. *J Biol Chem*. 1998; 273:24979–24982. [PubMed: 9737951]
23. Loria PM, Hodgkin J, Hobert O. A conserved postsynaptic transmembrane protein affecting neuromuscular signaling in *Caenorhabditis elegans*. *J Neurosci*. 2004; 24:2191–2201. [PubMed: 14999070]
24. Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD, Winter G. By-passing immunization. Human antibodies from V-gene libraries displayed on phage. *Journal of molecular biology*. 1991; 222:581–597. [PubMed: 1748994]

25. Mello CC, Kramer JM, Stinchcomb D, Ambros V. Efficient gene transfer in *C.elegans*: extrachromosomal maintenance and integration of transforming sequences. *Embo J*. 1991; 10:3959–3970. [PubMed: 1935914]
26. Nadanaka S, Kitagawa H. Heparan sulphate biosynthesis and disease. *J Biochem*. 2008; 144:7–14. [PubMed: 18367479]
27. Okkema PG, Harrison SW, Plunger V, Aryana A, Fire A. Sequence requirements for myosin gene expression and regulation in *Caenorhabditis elegans*. *Genetics*. 1993; 135:385–404. [PubMed: 8244003]
28. Pedelacq JD, Cabantous S, Tran T, Terwilliger TC, Waldo GS. Engineering and characterization of a superfolder green fluorescent protein. *Nature biotechnology*. 2006; 24:79–88.
29. Potter CJ, Tasic B, Russler EV, Liang L, Luo L. The Q system: a repressible binary system for transgene expression, lineage tracing, and mosaic analysis. *Cell*. 2010; 141:536–548. [PubMed: 20434990]
30. Praitis V, Casey E, Collar D, Austin J. Creation of Low-Copy Integrated Transgenic Lines in *Caenorhabditis elegans*. *Genetics*. 2001; 157:1217–1226. [PubMed: 11238406]
31. Sarrazin S, Lamanna WC, Esko JD. Heparan sulfate proteoglycans. *Cold Spring Harbor perspectives in biology*. 2011:3.
32. Smits NC, Kurup S, Rops AL, ten Dam GB, Massuger LF, Hafmans T, Turnbull JE, Spillmann D, Li JP, Kennel SJ, Wall JS, Shworak NW, Dekhuijzen PN, van der Vlag J, van Kuppevelt TH. The heparan sulfate motif (GlcNS6S-IdoA2S)₃, common in heparin, has a strict topography and is involved in cell behavior and disease. *The Journal of biological chemistry*. 2010; 285:41143–41151. [PubMed: 20837479]
33. Smits NC, Lensen JF, Wijnhoven TJ, Ten Dam GB, Jenniskens GJ, van Kuppevelt TH. Phage display-derived human antibodies against specific glycosaminoglycan epitopes. *Methods Enzymol*. 2006; 416:61–87. [PubMed: 17113860]
34. ten Dam GB, van de Westerlo EM, Smetsers TF, Willemse M, van Muijen GN, Merry CL, Gallagher JT, Kim YS, van Kuppevelt TH. Detection of 2-O-sulfated iduronate and N-acetylglucosamine units in heparan sulfate by an antibody selected against acharan sulfate (IdoA2S-GlcNAc)_n. *The Journal of biological chemistry*. 2004; 279:38346–38352. [PubMed: 15247295]
35. Thompson SM, Connell MG, van Kuppevelt TH, Xu R, Turnbull JE, Losty PD, Fernig DG, Jesudason EC. Structure and epitope distribution of heparan sulfate is disrupted in experimental lung hypoplasia: a glycobiological epigenetic cause for malformation? *BMC Dev Biol*. 2011; 11:38. [PubMed: 21672206]
36. van de Westerlo EM, Smetsers TF, Dennissen MA, Linhardt RJ, Veerkamp JH, van Muijen GN, van Kuppevelt TH. Human single chain antibodies against heparin: selection, characterization, and effect on coagulation. *Blood*. 2002; 99:2427–2433. [PubMed: 11895775]
37. van Kuppevelt TH, Dennissen MA, van Venrooij WJ, Hoet RM, Veerkamp JH. Generation and application of type-specific anti-heparan sulfate antibodies using phage display technology. Further evidence for heparan sulfate heterogeneity in the kidney. *J Biol Chem*. 1998; 273:12960–12966. [PubMed: 9582329]
38. Wei X, Potter CJ, Luo L, Shen K. Controlling gene expression with the Q repressible binary expression system in *Caenorhabditis elegans*. *Nat Methods*. 2012; 9:391–395. [PubMed: 22406855]
39. Wijnhoven TJ, Lensen JF, Rops AL, van der Vlag J, Kolset SO, Bangstad HJ, Pfeffer P, van den Hoven MJ, Berden JH, van den Heuvel LP, van Kuppevelt TH. Aberrant heparan sulfate profile in the human diabetic kidney offers new clues for therapeutic glycomimetics. *American journal of kidney diseases : the official journal of the National Kidney Foundation*. 2006; 48:250–261. [PubMed: 16860191]

Figure 1.

a. Schematic of the basic HS structure organized into domains with varying degrees of sulfation. Modifications and putative growth factor binding sites are indicated.

b. Schematic of the secreted scFv antibody fusion (inset) and the basic plasmid construct (1). The construct was designed in a modular fashion to allow for easy swapping of the heavy chains, targeting sequence, 3' UTR, fluorophores, and promoters. Two-headed arrows indicate alternative modules that could be exchanged with existing modules. Common restriction sites are indicated. NLS: nuclear localization signal.

c. Diagram of a transgenic worm expressing the antibody construct from specific cells (coelomocytes in this example). The antibody is secreted into the pseudocoelom where it diffuses and binds to heparan sulfate domains. Unbound antibody is taken up by the coelomocytes which absorb material from the pseudocoelom (9).

Figure 2.

a.-b. Epifluorescent micrographs of transgenic animals expressing an EW4G1::GFP (a) or LKIV69::GFP fusion (b) under control of the coelomocyte-specific *punc-122* (23) promoter show staining of the nervous system in *C. elegans*. In all panels, filled or open green arrow heads indicate the dorsal and ventral nerve cords, respectively and, magenta arrowheads indicate neuronal staining associated with the *C. elegans* nerve ring. No comparable staining was observed when a control scFv antibody fusion (MPB49::GFP) was expressed under the same conditions (1). The gut with characteristic vesicular autofluorescence is indicated. Scale bars indicate 25 μ m in all panels and p: pharynx.

c. Epifluorescent micrographs of transgenic animals expressing an HS3A8::GFP antibody fusion under control of the coelomocyte-specific *punc-122* (upper panel) and a presynaptic marker (mCherry::RAB-3 fusion) under control of a promoter specific for GABAergic neurons (middle panel) (Attreed & Bülow, unpublished). A merged image (lower panel) shows partial colocalization of HS3A8-specific HS epitopes with the presynaptic marker in GABAergic neurons.

d. Epifluorescent micrographs of transgenic animals expressing an HS4C3::DsRed2 antibody fusion under control of the coelomocyte-specific *punc-122* (upper panel) and a presynaptic marker (SNB-1::GFP) under control of a promoter specific for GABAergic neurons (middle panel) (*juIs1*, (20)). A merged image (lower panel) shows partial

colocalization of HS4C3-specific HS epitopes with the presynaptic marker in GABAergic neurons.

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