Generating transgenic reporter lines for studying nervous system development in the cnidarian *Nematostella vectensis*

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Abstract

Neurons often display complex morphologies with long and fine processes that can be difficult to visualize, in particular in living animals. Transgenic reporter lines in which fluorescent proteins are expressed in defined populations of neurons are important tools that can overcome these difficulties. By using membrane-attached fluorescent proteins, such reporter transgenes can identify the complete outline of subsets of neurons or they can highlight the subcellular localization of fusion proteins, for example at pre- or postsynaptic sites. The relative stability of fluorescent proteins furthermore allows the tracing of the progeny of cells over time and can therefore provide information about potential roles of the gene whose regulatory elements are controlling the expression of the fluorescent protein. Here we describe the generation of transgenic reporter lines in the sea anemone *Nematostella vectensis*, a cnidarian model organism for studying the evolution of developmental processes. We also provide an overview of existing transgenic *Nematostella* lines that have been used to study conserved and derived aspects of nervous system development.

Key words: transgenic reporter, *Nematostella*, Cnidaria, fluorescent protein, neurogenesis, microinjection

1 Introduction

Cnidarians are a group of morphologically diverse animals that includes corals, sea anemones and various types of jellyfish. They are the sister group to bilaterians [1] and as such occupy an informative position in the tree of life for understanding the early evolution of nervous systems. The relevance of cnidarians for the evolution of nervous systems has long been recognized, but only with the advent of molecular tools in the last ten to twenty years, it became possible to identify common and divergent features of cnidarian and bilaterian nervous system development [2-5].

Cnidarians are grouped into anthozoans and medusozoans, the latter typically being characterized by the presence of a pelagic medusa in their life cycle, which is generated asexually from polyps [6]. Anthozoans, in contrast, lack a medusa stage and exist only as sexually reproducing, sessile polyps. The nervous system of polyps contains areas of lower and higher density of neurons and can feature tracts of longitudinal neurites, but its overall organization is best characterized as a nerve net [2-5]. Free-swimming medusae display a much higher degree of concentration of neural elements [7, 8]. This is particularly evident in the rhopalia, light and gravity sensing structures located at the margin of the bell in scyphozoan and cubozoan jellyfish. Rhopalia can contain thousands of neural cells and contribute to the control of swimming behaviour [9, 10]. Cells of the nervous system in cnidarians have traditionally been grouped into sensory cells (including sensory-motor cells), ganglion cells (morphologically equivalent to interneurons) and cnidocytes (“stinging cells”). Morphological, immunohistochemical and gene expression analyses have shown that these three main groups consist of many subgroups, but the integration of morphological and molecular data to define these subgroups more precisely is still in its early stages [4, 11]. The development of the nervous system has been studied mainly in two groups of cnidarians, hydrozoans (part of the medusozoans) and anthozoans. In both groups, nerve cells develop in

a spatially distributed manner throughout the tissue. In hydrozoans, most neural cells are generated from interstitial stem cells (i-cells), a heterogeneous population of cells with mesenchymal appearance that includes multipotent cells [12-14]. Embryonically, i-cells originate from the endoderm before they can migrate to the ectodermal layer [15-17]. Anthozoans embryos do not possess cells that resemble interstitial cells at the morphological level. Neural cells first appear in the ectoderm and after gastrulation also in the endoderm [18]. Epithelial neural progenitor cells are located in both germ layers and give rise to sensory cells and ganglion cells [4, 19]. Cnidocytes (stinging cells), a sensory cell type used for predation and defense are formed exclusively in ectodermal tissue. Despite this uncommon, broad neurogenic potential, cnidarian neurogenesis shares many molecular features with bilaterian neurogenesis, e.g. the involvement of SoxB and bHLH transcription factors and the Notch and Wnt signalling pathways (though a role for Notch in early neurogenesis seems to be absent in hydrozoans, [20-24]). These observations now allow us to move beyond candidate gene approaches to obtain a detailed understanding of the molecular basis for the broad neurogenic potential of cnidarians during embryonic development and regeneration. A key technological progress for studying neural development and neural function in cnidarians was the establishment of stable transgenic lines [25-27]. The distributed organization of the nervous system in cnidarians makes it difficult or impossible to use the position of cells expressing particular genes (detected by in situ hybridization) as an indicator of their neural identity. Transgenic reporter lines overcome this problem by visualizing the morphology of cells expressing a transgene, for example membrane tethered fluorescent proteins. Moreover, they allow tracing the progeny of the cells after they terminated the expression of a gene of interest and they have been used to record neural activity via genetically encoded calcium sensors [28]. Successful use of transgenic animals has been reported for the hydrozoans *Hydra* and *Hydractinia*, and for the anthozoan *Nematostella* [25, 27, 29]. Here we focus on

Nematostella vectensis to summarize features of published "neural" *Nematostella* reporter lines and provide a protocol for the generation of transgenic lines. This protocol is based on the one published by Renfer and Technau, 2017 [26] which uses the I-SceI Meganuclease to obtain transgenic lines with good efficiency.

Available *Nematostella* reporter lines for the nervous system

NvSoxB(2)[19] The HMG box transcription factor *NvSoxB(2)* is expressed from blastula stages on in mitotic and non-mitotic cells scattered throughout the ectoderm and (after gastrulation) the endoderm. The transgenic reporter line revealed that these cells develop into sensory cells, ganglion cells and cnidocytes. Mitotic memOrange-positive cells can also be detected, but their number is significantly lower than that of mitotic cells expressing *NvSoxB(2)* mRNA. The *NvSoxB(2)* mRNA expressing cells thus include neural progenitor cells that generate a large fraction of the larval nervous system. Individual memOrange positive cells can be detected by immunohistochemistry (using anti-dsRed antibodies) from gastrula stage on; in living or unstained animals, however, individual cells can be distinguished only at planula stage.

NvElav1[18] Orthologs of the RNA binding protein Elav are often considered pan-neural markers in bilaterians. In *Nematostella*, the *NvElav1::memOrange* reporter line labels a population of sensory cells and ganglion cells in both germ layers, starting from gastrula stage and persisting in adult polyps. Double transgenic animals suggest that *NvElav1*::Cerulean-positive cells constitute a subpopulation of the *NvSoxB(2)*::memOrange-positive cells. While the *NvElav1* transgenics label a large number of morphologically heterogeneous neurons, co-labelling with antibodies against neuropeptides has shown that neither the transgenic line, nor the *elav1* transcript are pan-neural markers in *Nematostella*. Notably, the *NvElav1* transcript and the transgenic line do not label cnidocytes. The *Nematostella* genome encodes a second

elav gene, which is not part of the "neural" group of elav genes. It has so far not been possible to determine the mRNA expression pattern of *NvElav2*.

NvLWamide-like [30] Transcripts for the neuropeptide *NvLWamide-like* are first detected in the aboral half of gastrula stage embryos and from planula stage on in ecto-and endodermal cells, including the pharynx and (at later stages) the tentacles. Fluorescence derived from the *NvLWamide-like* ::mCherry transgene is visible in neurons from mid-planula stage on in the ectoderm and pharynx and subsequently in the endoderm. The number of *NvLWamide-like::*mCherry-positive cells is small compared to the number of *NvElav1*::memOrange cells, which allowed the identification of specific subsets of these cells. Interestingly, some of these subsets display only little variability in their neurite projection patterns and positions in the body column. This high level of stereotypy will likely be beneficial for studying the mechanisms that control the positioning of these neurons and the outgrowth of their neurites.

NvFoxQ2d [31] The transcription factor *NvFoxQ2d* is expressed in both mitotic and non-mitotic cells in the ectoderm, starting at gastrula stage. Mitotic cells are not found in the *NvFoxQ2d::memOrange* transgenic line. One explanation for this observation can be that the *NvFoxQ2d* mRNA expressing cells undergo a terminal division and that the maturation time of the fluorescent protein precludes their identification in the transgenic line. The *NvFoxQ2d*::memOrange-positive cells are excluded from the oral area of the planula and polyp and they display a rather uniform morphology that resembles sensory cells. While they have only short neurite-like processes, they can be co-labelled with an antibody against FMRFamide neuropeptides, supporting their neural nature.

NEP3 [32] *NEP3* encodes a toxin whose mRNA is expressed in scattered cells of the ectoderm and in the tentacles. As suspected, a *NvNEP3::mOrange2* transgene encoding a

fusion protein of the predicted signal peptide of NvNEP3 and the fluorescent reporter identified the *NvNEP3*-expressing cells as a large subpopulation of cnidocytes.

2 Materials

2.1 Animal maintenance and preparation of eggs

1. *Nematostella* Medium

    - sea water (artificial of filtered natural) diluted to 14-16ppt.

2. 3% Cysteine solution (L-cysteine, Sigma) in *Nematostella* medium, adjust pH with NaOH to 7.4.

3. Pasteur pipettes with smooth opening (see Note 1)

3. Petri dishes (see Note 1)

4. Injection dish with a low wall (see Note 2)

2.2 Injection solution

1. I-SceI meganuclease (5U/µl, New England Biolabs)

2. Alexa-conjugated Dextran MW10.000 (Life Technologies, see Note 3)

3. Nuclease-free water for molecular biology

2.3 Capillaries

1. Holding capillary (custom-made Vacu Tip, Eppendorf, outer diameter 110µm, inner diameter 60µm, angle 25°, limb length 500µm, round front surface)

2. Injection capillaries (made from GB100TF-10 borosilicate capillaries with filament, Science Products, Germany, see Note 4)

2.4 Equipment

1. Heating block

2. Puller for injection capillaries (Sutter P-97)

3. Inverted microscope with fluorescence lamp

4. Coarse and fine micromanipulators (MN-4 and MMO-202ND, Narishige)

5. CellTram vario (Eppendorf, see Note 5)

6. Microinjection pump (FemtoJet, Eppendorf)

3 Methods

3.1 Design and cloning of constructs

The cloning cassette of the vector that has been used for the generation of transgenic *Nematostella* is flanked by sites for the I-SceI homing endonuclease and it contains an SV40 polyadenylation signal following the ORF of a fluorescent reporter protein of choice [25, 26]. The use of Gibson Assembly® [33] for cloning is a good option to keep the distance between the regulatory elements and the sequence encoding the fluorescent protein minimal. The cloning is performed by standard procedures; plasmids can be prepared with midi-prep kits to achieve high purity (see Note 6).

3.2 Preparation of embryos

1. To induce spawning, expose separate boxes with male and female polyps to bright light and a temperature of 24 -25°C for 12 hours (overnight, see Note 7).

2. In the following morning, discard egg packages that have been laid overnight. Keep the boxes at the standard culture temperature (18 - 19°C) and collect freshly laid egg

packages after two hours. Fertilize the egg packages by incubating them for 20 min with an excess of medium from the box containing the male polyps.

3. Transfer the fertilized egg packages to a petri dish and incubate them for 20 min in the 3% Cysteine solution (pH7.4) on a rotating shaker (see Notes 8 and 9)

4. Collect the fertilized eggs and wash them in *Nematostella* medium 5 x for 2min, 1 x 5min.

5. Place the de-jellied eggs in the injection room (see Note 10).

3.3 Preparation of injection solution

Prepare the solution for injection in a 1.5ml tube. Incubate at 37°C for 20min, keep dark. Store on ice

1. I-SceI buffer (10x)	1.0µl
2. Dextran MW 10.000 (200ng/µl)	2.0µl
3. Plasmid (100ng/µl, see Note 11)	2.0µl
4. I-SceI (5U/µl)	2.0µl
5. Nuclease-free water	to 10µl

3.4 Transfer of zygotes to injection plate and preparation of capillaries

1. Fill the injection dish with *Nematostella* medium and place it on the stage of the injection microscope. Use a Pasteur pipette with smooth opening to place the fertilized eggs in the injection dish. Attempt to place them as a column (see Note 12).

2. Insert the holding capillary in the capillary holder connected to the Cell Tram. The distal part of the capillary should be parallel to the surface of the injection dish.

3. Use a microloader pipette tip to fill the injection capillary from the back end. Insert the pipette tip as deep as possible and release the injection solution close to the tip of the capillary.

4. Insert the injection capillary in the capillary holder (see Note 13).

5. Connect the capillary holder to the FemtoJet injection pump via the attached tube (see Note 14).

6. Use the microscope stage to move the embryos into the field of view, then use the coarse and the fine micromanipulators to move the two capillaries into focus.

7. Lower the injection capillary until the site at which you want to break it touches the bottom of the dish. Move the tip of the holding capillary on top of the injection capillary and gently move it down to open the injection capillary (see Note 15).

3.5 Injection

1. Open the shutter for the fluorescent light and use the “inject” or the “clean” button of the injection pump to release air from the tip of the injection capillary. Adjust the injection volume via the injection pressure and the injection time (see Note 16).

2. Adjust the “hold pressure” to avoid that medium from the dish is aspired into the injection capillary.

3. Use the CellTram to hold a zygote and the micromanipulator to move the needle. Inject and remove the needle before releasing the zygote (see Note 17).

4. Move the microscope stage to place the next zygote in front of the holding capillary.

3.6 Identification of transgenic animals

1. Transfer the animals with clearly visible fluorescent dextran from the injection dish to a petri dish.

2. Use a stereomicroscope or an inverted compound microscope to monitor the animals for expression of the fluorescent protein at the expected stage of development. Separate animals into groups according to the strength/breadth of expression (see Note 18).

3. Raise the animals according to standard culture protocols.

4. Identify the sex of the animals and cross them individually to wild-type polyps (see Note 19) to identify carriers of the transgene.

4 Notes

1. *Nematostella* eggs and embryos stick to fresh plastic ware and glass pipettes. To reduce stickiness, we fill fresh pipettes and dishes with *Nematostella* medium for at least one night before first use and re-use them when possible.
2. A low wall allows mounting the injection capillary with a low angle in relation to the surface of the injection dish. With a low angle, the egg is pressed towards the holding capillary during penetration of the injection capillary. As injection dishes we use the lids of Nunc 4-well plates.
3. The colour of the fluorescent dextran should be chosen according to the fluorescent protein of the construct.
4. The quality of the injection capillary is important for the survival of the injected embryos and the number of embryos that can be injected in one session. Capillaries that are too wide will damage the embryo and render the control of the injection volume difficult. If the capillary is too thin, it will bend when it comes into contact

with the egg and it gets clogged more easily. Parameters for pulling the injection needles need to be determined for each heating filament of the puller. The Pipette Cookbook is a good source of information available on the website of Sutter Instruments.

5. Note that simpler microinjection setups can be used for *Nematostella* [34].
6. The identification of putative regulatory elements is greatly aided by the availability of genome-wide maps of chromatin modifications [35] that can be used to predict promoters and enhancers. When using upstream sequences only, we place the reverse primer immediately upstream of the start codon to include the 5'UTR. For many genes, however, the first intron contains putative regulatory elements [35] and should be included in the construct. We have so far used putative regulatory regions of 1.4 – 6kb for generating stable transgenic lines (unpublished data). Congruence of endogenous and transgene-derived expression can be tested by fluorescent double in situ hybridization with probes for the ORF of the fluorescent protein and the gene of interest.
7. In our feeding regime (*Artemia* nauplii 5 x per week), spawning of *Nematostella* polyps can be induced every 14 – 21 days for years. Shorter intervals (7 days) can be sustained for a few weeks, but fecundity tends to decrease thereafter. Protocols for *Nematostella* culture conditions and the induction of spawning have been published [36-38].
8. After fertilization, use a stereomicroscope to select egg packages in which the eggs are round and have a rather uniform diameter. Differences in egg size require repeated adjustment of the position of the capillaries during injection.
9. De-jellying by cysteine is an important step. If the eggs are not sufficiently de-jellied, both the holding and injection capillary will stick to the jelly and get clogged. If the

eggs are exposed to the cysteine solution too long, the survival rate decreases and more embryos display developmental abnormalities.

10. Injection can be done until the blastomeres are separated, this is often the 4-cell stage. Lower temperature delays development and allows more time for injection. We cool the injection room to 16-18°C.
11. The plasmid concentration that can be used for injection varies with the injection volume and with the quality of the plasmid preparation. A concentration of 20ng/µl is a good starting point, but the concentration can be increased as long as developmental defects and mortality remain limited.
12. To align the eggs in a column, they need to be released slowly into the injection dish. This, however, increases the risk of them sticking to the glass or plastic of the pipette.
13. Move the capillary into the holder carefully until it touches the O-ring.
14. Refer to the user manual of the injection pump for detailed description.
15. The opening of the injection capillary with the holding capillary can be time-consuming and the exact point of breakage is difficult to predict. The part of the holding capillary that is furthest down has to be used for breaking; otherwise, the holding capillary itself might be damaged. Depending on the angle in which the holding capillary is mounted, the bending point (the “knee”) of the holding capillary might be furthest down. In this case, the desired break point has to be placed under the “knee”. Alternatively, fine forceps can be used to cut the injection capillary.
16. Different labs use different volumes for the injection. The exact volume is difficult to determine because the fluorescent dextran diffuses very quickly throughout the egg. Injection into drops of oil to determine the injected volume is also difficult, because good injection capillaries tend to be too thin and flexible to be inserted into the oil. We

are trying to inject a drop of about ¼ of the diameter of an egg. With an average egg diameter of 230µm, this injection volume corresponds to approximately 100pl.

17. During the injection session, we keep both the shutters for the fluorescent and the transmitted light open. This allows control of the injected volume throughout the injection session. We have not observed any aversive effects of the excitation of fluorescent tracer.
18. The best time point for the screening of the injected animals depends on the injected construct and the expected pattern. In F0 planulae, we often observe expression that does not match the expected pattern, but this is no longer the case in primary polyps and it is not present in F1 planulae. When possible, we sort the F0 animals at primary polyp stage (10dpf), when they don't swim anymore. However, for genes that are expressed only at very early developmental stages, the fluorescent signal might no longer be detectable at primary polyp stage. The F0 animals display mosaic expression and we sort them according to the area in which expression is visible, with the assumption that a bigger area of expression increases the likelihood of germline transmission. Ideally, we raise ca 100 F0 polyps that show expression of the transgene. It should be noted, though, that for genes that are expressed distant from the mesenteries (e.g. in tentacles) transgene expression in F0 animals may not be a good selection criteria, as non-fluorescent, but transgenic patches may well reside in the germline, while fluorophore-expressing animals may not harbour transgenic patches in the germline. In such cases, it is best to simply raise all or a random selection of embryos to the next generation.
19. With the described method, integration of the transgene can occur at different positions in the genome. Spatial and temporal aspects of the expression of the transgene can thus be affected by the integration site. It is therefore desirable to

generate transgenic lines from more than one F0 animal to allow comparison of the observed expression.

20. The sex of the adult polyps can only be determined by inducing them to spawn individually.

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Figures

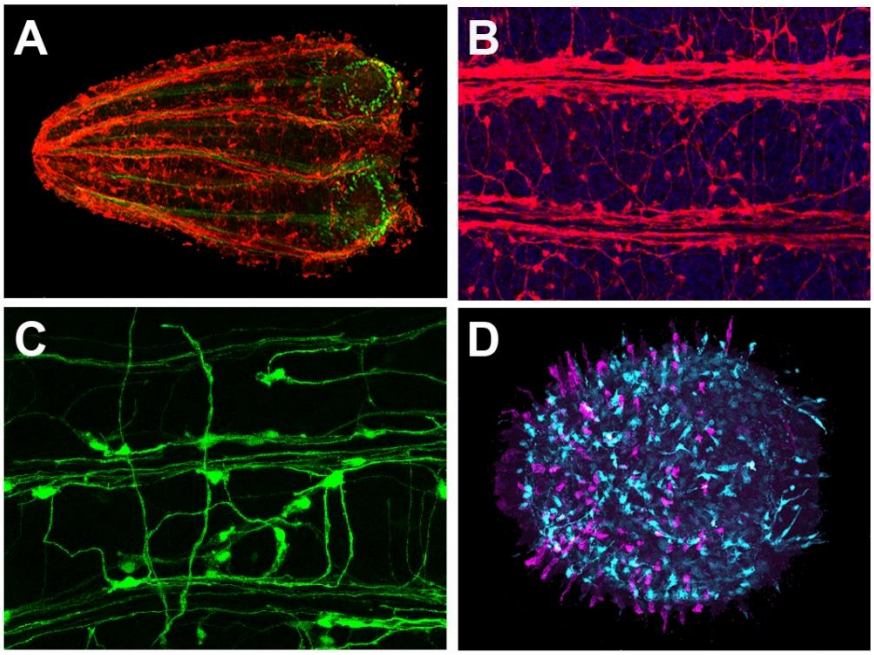

Figure 1 Transgenic *Nematostella* reporter lines. (A-C) Elav1::mOrange, detected by an anti-dsRed antibody and pseudocoloured. (A) Primary polyp (8 dpf), with aboral pole to the left, F-actin is stained with Phalloidin (green). (B, C) Body wall of 3-week old polyps. (B) is a F1 fully transgenic animal, note the two tracts of neurites that run along the oral-aboral axis (left to right). Nuclei are stained with DAPI (blue). (C) is a mosaic F0 transgenic animal that has been injected with the reporter plasmid. Fewer cells are labelled, allowing better resolution of individual neurons. (D) Elav1::cerulean (cyan), FoxQ2d::mOrange (magenta) double positive planula with aboral pole to the left.There is no overlap of the two neural cell poulations.

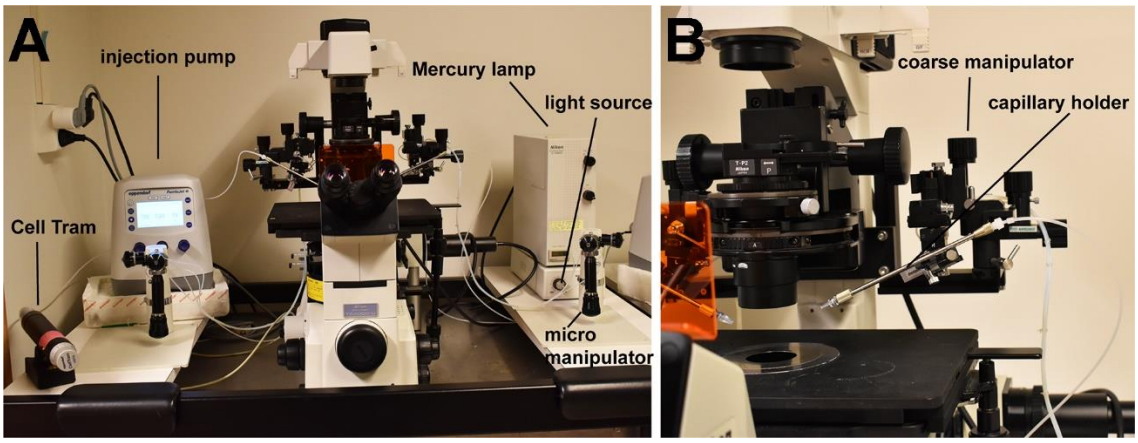

Figure 2 Microscope set-up for *Nematostella* microinjections. (A) Overview; the injection set-up is mounted on an inverted microscope equipped with a mercury lamp. The injection pump is connected to the injection capillary (on the right side of the microscope), the Cell Tram to the holding capillary (on the left side). A joystick operates the micromanipulator. Injection is triggered with a foot pedal or a by mouse click (not on the image). (B) The coarse manipulator is mounted on the microscope.

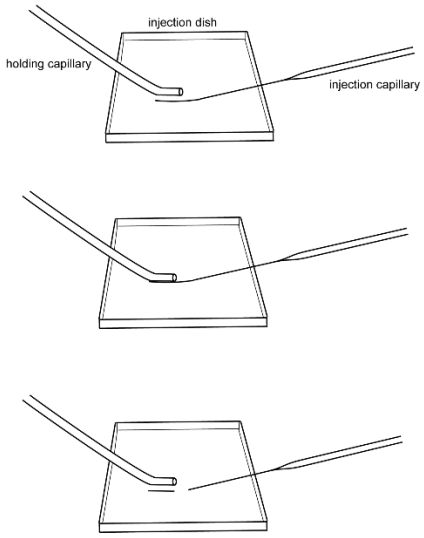

Figure 3 Opening of the injection capillary. Schematic depiction of the opening of the injection capillary with the holding capillary. The injection capillary is lowered to touch the bottom of the injection dish (point of contact is indicated by the bend of the injection capillary). The holding capillary is lowered to break the injection capillary (middle panel).