HuC-eGFP mosaic labelling of neurons in zebrafish enables in vivo live cell imaging of growth cones

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Abstract

The field of axon guidance is taking advantage of the powerful genetic and imaging tools that are now available to visualise growth behaviour in living cells, both in vivo and in real time. We have developed a method to visualise individual neurons within the living zebrafish embryo which provides exceptional cellular resolution of growth cones and their filopodia. We generated a DNA construct in which the *HuC* promoter drives expression of eGFP. Injection of the plasmid into single cell fertilised zebrafish egg resulted in mosaic expression of eGFP in neurons throughout the developing embryo. By manipulating the concentration of injected plasmid, it was possible to optimise the numbers of neurons that expressed the construct so that individual growth cones could be easily visualised. We then used time-lapse high magnification widefield epifluorescence microscopy to visualise the growth cones as they were exploring their environment. Growth cones both near the surface of the embryo as well as deep within the developing brain of embryos at 20 hours post fertilisation were clearly imaged. With time-lapse sequence imaging with intervals between frames as frequent as one second there was minimal loss of fluorescence intensity and the dynamic nature of the growth cones became evident. This method therefore provides high magnification, high resolution time-lapse imaging of living neurons in vivo and by use of widefield epifluorescence rather than confocal it is a relatively inexpensive microscopy method.

Key words: axon, filopodia, embryo, time-lapse, growth cone, telencephalon

1. Introduction

During development of the nervous system axons are often required to traverse a virgin microenvironment in order to precisely connect with distant targets. To accomplish this task, the growth cones of the axons need to differentiate between numerous chemoattractive and chemorepulsive guidance cues. The effect of any cue on a growth cone is difficult to predict as many responses are dependent upon the specific cellular context and extracellular environment. While current analyses have proven useful in establishing a general idea of the function of certain guidance molecules (see review by Hocking et al. 2012), new techniques in imaging and live cell labelling allow for more in depth and comprehensive studies to be performed.

The dynamic activity of growth cones within the living animal can only be clearly understood by time-lapse microscopy imaging. The zebrafish is an excellent model for nervous system development as its embryos are transparent and the genetic tools are providing the ability to visualise specific cells as they are interacting with other cells within the living embryo. Plasmids that encode genetic constructs that encode fluorescent reporters can be injected into the fertilised embryos which will generate mosaic expression of the constructs (Culp et al. 1991; Feng et al. 2000). By titering the amount of plasmid injected, the expression can be varied from large numbers of cells to small numbers of cells (Feng et al. 2000). The advantage of such an approach is that single cell analysis can be performed that will reveal the dynamic nature of individual growth cones as they are forming or migrating along a neural pathway (Jontes et al. 2000). In contrast, if all neurons express the fluorescent reporter it would be difficult if not impossible to achieve sufficient resolution of the individual axons (Connor et al. 2005). Despite the advantages of zebrafish animal model there are still very few reports analysing dynamic near-real time growth cone behaviour at a single cell level and at high resolution in vivo.

We have developed a method to visualise individual growth cones within the living zebrafish embryo. We generated a construct in which the promoter for *HuC* (Park et al. 2000) drives expression of enhanced green fluorescent (eGFP) protein in neurons. Zebrafish *HuC* is an RNA binding protein of *Drosophila* *elav* family and has 89% homology to human *HuC* protein (Kim et al. 1996). It is considered an early neuronal marker in developing zebrafish (Kim et al. 1996). Injection of the *HuC-eGFP* construct at the one cell stage yielded expression of eGFP in a low number of neurons throughout the embryo. We have used high magnification widefield epifluorescence time-lapse microscopy with imaging times as frequent as one frame per second to

visualise the dynamic behaviour of growth cones. This method provides excellent resolution and is a relatively inexpensive method for studying the behaviour of axons.

2. Materials and Methods

2.1 Fish Maintenance

Zebrafish (*Danio rerio*) were maintained at the animal house facility in the School of Biomedical Sciences, The University of Queensland, on a 14-hour light/10-hour dark cycle. Embryos were naturally spawned through light-induced mating and raised at 28.5°C in embryo medium (Westerfield 1994). Embryos were staged according to Kimmel et al. (1995).

2.2 Plasmid construction and microinjection

A plasmid was designed in which the promoter of zebrafish *HuC* gene drove expression of eGFP. A 3.2kb DNA fragment of 5' UTR sequence of zebrafish *HuC* gene (Park et al. 2000), containing the minimal promoter for neuron specific expression was amplified from genomic DNA using the following primers: HuC F: 5'–GAATTCACCTAATTTGAAT-3' and HuC R: 5' –TCTTGACGTACGTACAAAGATG-3'. The PCR product was gel purified and TA cloned into the pGEM-T vector (Promega Corporation, Madison, WI). The HuC promoter was excised from pGEM-T using flanking EcoRI sites and cloned directly into the EcoRI site of peGFP-1 vector (Clontech) to generate the HuC:eGFP construct. Approximately 0.5 – 1 nL of solution containing 37.5 pg of DNA was injected directly into the cytoplasm of the cell. Microinjection was performed using a gas driven microinjection apparatus (PV830 Pneumatic Picopump, World Precision Instruments, Sarasota, FL, USA). At each injection session, 20-30 embryos were injected; up to 80% of embryos then showed eGFP expression in neurons.

2.3 Immunohistochemistry

Embryos were fixed for 20 min in 4% paraformaldehyde, washed for 2 × 5 min in phosphate buffered saline, partially cleared in 70% glycerol with 0.1% sodium azide and stored in 100% methanol. The whole brains of zebrafish embryos were dissected and washed for 3 × 5 min in Tris buffered saline and 0.3% Triton X-100 (TBST) and subsequently transferred to blocking solution (2% Bovine serum albumin in TBST) and incubated for 1 h at room temperature. The blocking solution was removed and replaced with solution containing mouse monoclonal IgG anti-acetylated α -tubulin monoclonal antibody (1:200; ICN Biochemicals, Costa Mesa, CA), which binds to all axons. Brains were incubated for 15 h at 4°C and washed for 3 × 5 min in TBST. To visualize binding, embryos were reacted with a monoclonal goat anti-mouse IgG (α -chain specific) conjugated to Texas red (1:50; Vector Laboratories, Burlingame, CA). Embryonic brains were dissected and mounted in fluorescent

mounting media (200 mg/ml propyl gallate, 5% ethanol, 95% glycerol, 25 mM sodium bicarbonate, pH 9.6) between two coverslips, and serial optical sections were collected with a Bio-Rad MRC-1024 laser scanning confocal microscope coupled to a Zeiss Axioplan microscope. Scans were compiled by using Bio-Rad confocal assistant program.

2.4 Time-lapse Microscopy

At 18 hours post fertilization (hpf) injected embryos were screened for neuronal GFP fluorescence. Embryos were anaesthetized in 0.01% Tricane (3-amino benzoic acid ethylester) (Sigma) and mounted on glass slides in 1% low melting temperature agarose. Time-lapse images were captured using an Olympus BX51 compound microscope using widefield epifluorescence. Low power images of the whole embryo was captured using a dry 2X Plan N objective (NA 0.06), images of axons were captured using either a dry 40X Plan Apo objective (NA 0.95) or an oil 100X DPlan objective (NA 1.25). Exposure times varied depending on the specimens but were typically in the range of 50-300 ms. Time-lapse sequences were collected using the Evolution OEi microscope camera and Image Pro/Scope Pro deconvolution software (Media Cybernetics Image Analysis Software, Silver Spring, MD, USA), with images taken at intervals ranging from 1-10 seconds for periods up to one hour. Images were cropped, brightened and gamma adjusted to provide detail of filopodia using Adobe Photoshop CS5 with the entire field of view being treated uniformly. Figures were compiled using Adobe Illustrator CS5 (Adobe Systems Incorporated). Movies of imaging sequences were generated using ImageJ 1.43u and saved in avi format. Real time movies were generated with each frame being displayed at the same time interval that they were originally captured; time-lapse movies were generated with frames being displayed at four frames per second with each frame having been captured at intervals of 1-10 seconds.

2.5 Quantification of fluorescence stability

The mean gray level of the growth cone over the imaging sequence was determined using Image J. The area of interest (AOI) was selected to ensure that the growth cone remained within the confines of the AOI and the mean gray level was measured for each frame.

3. Results

3.1 *HuC-eGFP labels neurons in the embryonic zebrafish*

The transgene construct *HuC-eGFP* was used to express eGFP in neurons and their axons. Plasmid expression vectors that are microinjected into zebrafish embryos at the one cell stage are often mosaically expressed (Hocking et al. 2012). The zebrafish embryo develops rapidly (Fig. 1A) and by 18 hours post fertilisation (hpf) the development of nervous system is well underway. The unequal distribution of injected plasmids from mother to daughter cells as well as stochastic expression of eGFP results in mosaic labelling of neurons throughout the nervous system. Embryos that had been injected with 37.5 pg of *HuC-eGFP* showed a restricted number of dispersed neurons that were labelled by eGFP and which also enabled distinct resolution of the axons, growth cones and filopodia. Thus, the mosaic expression of eGFP in a small number of neurons provided the necessary single cell resolution that would not be possible if larger subpopulations of neurons expressed the reporter molecule. The efficiency of injection and expression of the plasmid was high and up to 80% of embryos expressed eGFP in neurons and thus sufficient numbers of embryos could be screened for eGFP expression in target neurons.

We initially examined Rohon-Beard sensory neurons whose perikarya are located in the dorsal spinal cord. These neurons project peripheral axons into the skin and are responsible for the touch response of early embryos (Saint-Amant 2010). The cell bodies of the Rohon-Beard neurons strongly expressed eGFP making them easily identifiable. The peripheral axons of these neurons uniformly expressed eGFP along their entire length including the growth cones and filopodia (Fig. 1B). At higher magnification, the morphology of the growth cones was clearly distinguishable above the background fluorescence of the surrounding cells (Fig. 1C). The initial formation of the neural tracts is well established by 1 hpf (Fig. 1B) and *Huc-eGFP* labelled neurons within deeper regions of the brain were present at this stage. Some neurons within the dorsal rostral cluster of the telencephalon were labelled by eGFP while other neurons were unlabelled but could be detected by immunostaining with antibodies against acetylated alpha tubulin (Fig. 1E-F). Confocal imaging of growth cones very high resolution (Fig. 1F), but widefield epifluorescent imaging of growth cones also provided high resolution albeit without such focal depth (Fig. 1B-C).

3.2 *Time-lapse imaging of *HuC-eGFP* growth cones*

To perform time-lapse imaging of the axons within the living zebrafish, embryos were anaesthetized and immobilised in agarose to reduce movement. Widefield epifluorescence time-lapse microscopy of a Rohon-Beard neuron enabled clear visualisation of the axons, growth cones and filopodia with minimal loss of fluorescence intensity over the imaging period (Fig. 2, Movie 1). While skin did not express the reporter eGFP some cells could be visualised by background epifluorescence emissions (Fig. 2). This fortuitous staining provided convenient landmarks to assess axon growth and to reveal underlying cell-cell interactions which would have otherwise remained hidden. Individual frames from the movie revealed the remarkable rate of elongation of the axons (Fig. 2). One axon was observed to extend and consolidate a single filopodia approximately 20 μm from its growth cone across the surface of fluorescent cells within 1 minute and 35 seconds (solid arrows, Fig. 2A-C). This filopodia was subsequently converted into an axon as the growth cone progressively moved anterogradely (solid arrows, Fig. 2D-F; Movie 1). At the same time an axon branch was observed retracting from a cell (arrowhead, Fig. 2B-D) while multiple short filopodia at the leading edge of another axon from the same neuron were observed exploring and extending over the surface of another cell (unfilled arrows, Fig. 2A-F). This behaviour highlights the ability of a single neuron to exhibit highly selective responses within a similar microenvironment. The ability of axons from the same neuron to respond differently and at the same time to these cells raises interesting questions regarding the molecular nature of the guidance cues and the spatial regulation of the subcellular response to these cues.

Increasing the length of the time-lapse imaging sequence time from approximately 5 minutes to 10 minutes (Fig. 3, movie 2) had no detrimental effect on the dynamic activity of axon branching, filopodial extension and retraction and growth cone advance. It should be noted that each of the axons were equally labelled by eGFP which confirmed that this reporter was able to capture growth activity across the whole cell. This longer analysis time of a single Rohon-Beard neuron highlighted multiple self-recognition events as single axons interacted with other axons from the same neuron. It was clear that these axons did not grow over each other and instead typically retracted upon contact which probably contributed to the continual spread of axon branches within the periphery. This advance, recognition and then retraction occurred rapidly (in some cases within 2.5 minutes; see arrows in Fig. 2B-D). Interestingly, the recognition event can last long enough for multiple branches to form (see arrows, Fig 2C and F).

3.3 Real time imaging of growth cones

Due to the stability of fluorescence during widefield epifluorescence microscopy imaging over extended periods, we next examined growth cones using high magnification imaging with images captured every second. Imaging of a single Rohon-Beard growth cone at high magnification (Fig. 4, Movie 3) revealed the complex morphology of the leading edge of an axon *in vivo*. Rather than assuming the club- or torpedo-like shape as often represented in static images of growth cones from fixed tissue (Bovolenta and Mason 1987), the living growth cone can be an elongated structure possessing many distinct and dynamic regions of membrane growth. During a 60 second imaging period, some filopodia contacted and adhered to each other (arrows, Fig. 4A-C) while in other regions numerous filopodia emerged (arrowheads, Fig. 4D-E). When the image sequence is played as a movie with each frame being displayed at the same rate it was captured (one frame/second), the rapid and dynamic behaviour of the Rohon-Beard growth cone is clearly evident (Movie 3). The intensity of fluorescence of the growth cone was determined by measuring the mean gray level of the growth cone and was found to decrease by only 7% during one minute of the sequence (Fig. 4F). With longer imaging intervals the eGFP-labelled neurons could be imaged for many hours, however the rapid development of the embryo often necessitated altering the field of view as the cellular mass changed so quickly. The eGFP-labelled neurons were easily visible and could be imaged in embryos up to 48 hpf; after this time the development of the skin reduced the resolution and clarity of the imaging.

Neurons that were labelled by the *HuC-GFP* plasmid were also present deep within the brain and widefield epifluorescence imaging was able to provide detailed resolution of the growth cone (arrow, Fig. 5A) as well as filopodia dynamics (Movie 4). Frequent imaging of the neurons within the deeper layers was easily achieved. In one example, the growth cone of a neuron from within the telencephalon was imaged every two seconds for over 8 minutes (Fig. 5, Movie 4). The movement of the filopodia was evident with filopodia retracting (arrowheads, Fig. 5E-G) or extending (arrow with tail, Fig. 5G-H) from the same region of the growth cone. When the image sequence is played as a movie with each frame being displayed at the same rate it was captured (one frame every two seconds), the real time rapid and dynamic behaviour of the growth cone within the telencephalon is clearly evident (Movie 4). The intensity of fluorescence of the growth cone was maintained throughout the imaging period (Fig. 5I).

4. Discussion

Time-lapse imaging of axons and their growth cones provides a wealth of information about the dynamic behaviour that occurs during development. Injection of a plasmid consisting of the promoter for the neuron-specific *HuC* gene (Park et al. 2000) driving expression of eGFP was found to generate mosaic labelling of neurons and their axons in the embryonic zebrafish nervous system. The use of the *HuC-eGFP* construct resulted in excellent resolution of the growth cones both near the surface of the embryo and within the deeper telencephalon. The expression of eGFP was uniform within each neuron including expression within multiple axons, axon branches, growth cones and their filopodia.

The eGFP fluorescence levels were stable and showed minimal loss of intensity over extended imaging periods with detailed morphology being maintained throughout imaging sequences with frame rate captures as frequent as one frame per second for at least 10 minutes. The *HuC* promoter has previously been used to generate constructs driving expression of kaede protein in zebrafish (Sato et al. 2006). The kaede protein can be photo converted from green to red to achieve visualisation of cells that have been activated. However, activated kaede protein has a limited life span and thus the cells need to be reactivated so that differential visualisation can occur. The *HuC* promoter has also been successfully used to drive expression of TdTomato to study afferent neurons of the lateral line (Faucherre et al. 2009). Mosaic expression of eGFP has been previously used to visualise neurons in the developing zebrafish using various promoters (Conway et al. 2006). In our hands the *HuC* promoter provided excellent mosaic expression and eGFP proved to be particularly stable during extended epifluorescence live cell imaging. Other promoters such as neurogenin1 (Andersen et al. 2011), the islet-1 promoter (Higashijima et al. 2000), and the *mnx1* enhancer (Zelenchuk and Bruses 2011) have been used to generate transient transgenic fish to visualise various neurons during development. The utility of these different promoters depends on the onset and extent of expression in the neuronal populations of interest.

The majority of live cell imaging studies use confocal imaging to visualise the neurons and growth cones, particularly at high magnification (e.g. Andersen et al. 2011; Choi et al. 2010; Martin et al. 2010; Jontes et al. 2000), but epifluorescence imaging has also been successfully used at lower magnification (Wolman et al. 2008). Here we have demonstrated that excellent resolution and focal depth can be obtained using high magnification widefield epifluorescence imaging. Spinning disk confocal imaging offers excellent resolution and speed, but access to such imaging hardware can be limited for many laboratories. The purchase cost of a

widefield epifluorescence microscopy is considerably less than a confocal microscope (5-10 times less depending on the models). In addition, the operating costs of widefield epifluorescence scopes are a fraction of the operating costs for confocal microscopes since expensive lasers need to be regularly replaced on confocal microscopes. Confocal microscopes are often in high demand at many institutes which lead to restricted availability and with time-lapse imaging of live embryos it is critical that the imaging is performed on time. Thus, the option of a dedicated time-lapse imaging widefield epifluorescence microscope may satisfy budget constraints and solve availability issues.

In this report we have demonstrated that the use of the *HuC-eGFP* construct in living zebrafish embryos together with high magnification widefield epifluorescence imaging provides excellent resolution of growth cones and their filopodia. We have used this method to generate movies which clearly exhibit the dynamic behaviour of growth cones. When played in real time the relatively rapid responses of the filopodia within the three-dimensional environment of the developing embryo is clearly evident. This inexpensive option of high magnification, high resolution live cell imaging would be attractive to many laboratories and when combined with knockdown or mutations of molecules of interest would provide considerable information about the dynamics of growth cone behaviour.

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Figure legends

Figure 1. HuC-eGFP mosaically labels neurons in zebrafish embryos. (A) By 20 hours post fertilisation, the neural pathways are being established in the zebrafish embryo. Time-lapse imaging of eGFP labelled neurons was performed on dechorionated and immobilised living embryos. (B) A Rohon-Beard neuron was labelled by HuC-eGFP and provided excellent visualisation of the axons and growth cones. The cell body of the neuron is very bright and indicated by the arrow. Another neuronal cell body is indicated by the arrowhead, its axons are not visible. (C) Epifluorescent imaging provided good contrast between the eGFP-expressing axons and the unlabelled cells underlying the axons. (D) The zebrafish forebrain at 20 hpf immunostained with acetylated alpha-tubulin antibodies. The embryo was imaged using confocal microscopy with scanning through one lateral half of the embryo. The orientation of the head is the similar to that shown in A. Asterisk indicates the dorsal rostral cluster of neurons that give rise to axons that form the supra optic tract shown in E. (E-F) Two neurons within the dorsal rostral cluster were labelled by HuC-eGFP (green) while other neurons and their axons were unlabelled by HuC-eGFP but were visualised by acetylated alpha-tubulin antibodies (red). Scale bar is 300 μm in A, 40 μm in B,E-F, 20 μm in C, 130 μm in D.

Figure 2. Rohon-Beard axons interact with surrounding cells. Time-lapse imaging using epifluorescence illumination provided contrast between the eGFP-expressing neurons and the surrounding unlabelled cells. The growth cones (arrows) of eGFP-expressing Rohon-Beard axons were seen to interact with unlabelled adjacent cell bodies that were visible by background autofluorescence. The unfilled arrow shows a growth cone that explored a cell; the filled arrow shows a growth cone that extended over a cell. A branch (arrowhead) of an axon interacted and then retracted from a cell. Asterisk in D shows a melanocyte. Time is in minutes and seconds. Scale bar is 30 μm . See Movie 1 for full time-lapse sequence.

Movie 1. Time-lapse movie of eGFP-expressing Rohon-Beard axons interacting with surrounding unlabelled cells. Select frames are shown in Figure 2. Frames were captured every 5 seconds. Time is shown in minutes and seconds.

Figure 3. Interactions of Rohon-Beard axon growth cones. Time-lapse sequence of branches of a single Rohon-Beard neuron reveal the interactions of filopodia as they contact adjacent branches (compare arrowheads in D,

E, F; arrows in B, C, D). Lower power view of the neuron is shown in Fig. 1B. Time is in minutes and seconds. Scale bar is 20 μm . See Movie 2 for full time-lapse sequence.

Movie 2. Time-lapse movie of growth cones of eGFP-expressing Rohon-Beard axons. Select frames are shown in Figure 3. Frames were captured every 10 seconds; time sequence misses 3 frames between 4:40 and 5:20 and 2 frames between 8:30 and 9:00 due to adjustments to the microscope being made during acquisition which prevented images being captured. Time is shown in minutes and seconds.

Figure 4. Growth cone morphology of a Rohon-Beard axon. Time-lapse imaging of an elongated growth cone with numerous filopodia reveals its dynamic activity with filopodia rapidly protruding (arrowheads, D-E) and making contact with each other (arrow A-C). Images in the original time-lapse sequence were captured every second; frames shown are 10 seconds apart; time is in minutes and seconds. Scale bar is 10 μm . See Movie 3 for part of imaging sequence shown in real time. (F) Change in mean gray level of growth cone over one minute imaging sequence with measurement made for each one second interval frame.

Movie 3. High magnification real time movie of a growth cone from an eGFP-expressing Rohon-Beard neuron. Frames were captured every second and are displayed every second (real time). The movie shows a short extract from the entire sequence; select frames from the entire sequence are shown in Figure 4. Time is shown in minutes and seconds.

Figure 5. Growth cone morphology within the presumptive telencephalon. A time-lapse sequence of a growth cone showing the dynamic nature of the filopodia. Filopodia that protrude from the central domain (arrow) of the growth cone rapidly extend (arrow with tail G-H) and retract (arrowhead, E-G). Asterisk in A indicates an axon that is out of the focal plane. Frames shown are one minute part; time is in minutes and seconds. Scale bar is 10 μm . See Movie 4 for part of the imaging sequence shown in real time. (I) Change in mean gray level of growth cone over two minutes of the imaging sequence with measurements made for each two-second interval frame.

Movie 4. Real time dynamic movement of an eGFP-expressing growth cone within the telencephalon. Frames were captured every 2 seconds and are displayed every 2 seconds (real time). The movie shows a short extract

from the entire sequence; select frames from the entire sequence are shown in Figure 5. Time is shown in minutes and seconds.