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Research Report

Fluorescent tagged analysis of neural gene function using mosaics in zebrafish and *Xenopus laevis*

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ABSTRACT

An important question in the neurosciences is the role of specific gene expression in the control of neural morphology and connectivity. To address this question, methods are needed for expression of exogenous genes in a subset of neurons. This limited and mosaic expression allows the assessment of gene expression in a cell autonomous fashion without environmental contributions from neighboring expressing cells. These methods must also label neurons so that detailed morphology and neural connections can be evaluated. The labeling method should label only a subset of neurons so that neuronal morphology can be viewed upon a non-stained background, in a Golgi staining fashion. Here, we report methods using plasmids called pTAGUM (tagged analysis of genes using mosaics) that accomplish these goals. These methods should prove useful for the analysis of neural gene function in two important model organisms, the zebrafish and *Xenopus laevis*.

1. Type of research

Over a century ago, Camillo Golgi developed the silver bichromate "black reaction" that revealed the fine morphology of individual neural processes (Golgi, 1898). Ramón y Cajal used this technique to confirm the cellular nature of the nervous system and thus founded the field of neurobiology (Ramon Y Cajal, 1892, 1911, [1908] 1954). The utility of this technique lies in its inefficiency. Only a few neurons stain allowing the detailed visualization of all processes of an individual neuron upon a clear background. If the Golgi method stained all neurons, the dense interweaving of neuronal processes would prevent the visualization of individual neuronal morphology.

A molecular equivalent of Golgi stain is the mosaic expression of fluorescent protein in neurons and has been achieved in different organisms by a variety of approaches including electroporation, transfection, microinjection, and FLT/FLP recombination (Teh et al., 2003; Downes et al., 2002; Lee and Luo, 1999; Koster and Fraser, 2001). Curiously, expression of fluorescent proteins under the control of neurospecific promoters in transgenic mice often leads to mosaic expression in a subset of neurons (Feng et al., 2000). This mosaic expression is inherited, characteristic of the transgenic line, and likely a consequence of differing integration sites and the chromatin context in which they reside. This "Golgi-like" staining has been exploited to study neuron projections and axon behavior in the mouse (Bishop et al., 2004).

Many of these mosaic expression approaches allow the co-expression of mutant or exogenous proteins in these neurons to assess the cell autonomous consequences of gene expression during neural differentiation and pathfinding. Therefore,

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mosaic expression allows the detection of mutant cells or mis-expressing cells within a wild-type background. Expressing neurons can be easily identified from neighboring non-expressing neurons and surrounding cells. One can obtain both qualitative and quantitative data about the effects of exogenous gene expression on neural morphology, differentiation, and function. Fluorescent protein expression also allows the visualization of neurons and their processes in living embryos (Zito et al., 1999; Van Den Pol and Ghosh, 1998; Murray et al., 1998; Knobel et al., 1999; Dynes and Ngai, 1998; Chalfie et al., 1994).

Microinjection of plasmids into early fish and frog embryos provides a simple method to achieve mosaic expression. Plasmids are replicated episomally and persist throughout embryo development. Expression in these founder individuals is mosaic, and, in one model organism, the zebrafish, expression from tissue-specific promoters often gives faithful expression. In a previous study analyzing the zebrafish GATA2 promoter, wide neural-specific expression of EGFP was conferred by a construct, nsP5-GM2, containing the GATA2 minimal promoter plus a fragment containing a neural-specific enhancer (Meng et al., 1997). This study did not analyze the specific neural types expressing this transgene in depth nor was transient expression optimized. The neural expression conferred by the GATA2 enhancer suggested that this promoter might be excellent for the tagged analysis of genes in neurons using mosaics.

Here, we report a refinement of the zebrafish mosaic technique for neural expression of EGFP. Our DNA construct, called pTAGUMGATA2 (tagged analysis of genes using mosaics), uses the GATA2 minimal promoter and neural enhancer regions to drive strong mosaic enhanced green fluorescent protein (EGFP) expression to a large variety of neuronal types during early zebrafish development. This construct allows the expression of in frame EGFP fusion proteins such that the effects of exogenous gene expression can be rapidly detected.

We have also tested the utility of this construct in another model organism, *Xenopus laevis*. We reasoned that the zebrafish GATA2 promoter and neural enhancer might also function in *Xenopus* since *Xenopus* promoters often function with similar tissue specificity in zebrafish (Amsterdam et al., 1995; Lin et al., 1994). Unfortunately, the pTAGUMGATA2 construct does not give neural-specific expression in *X. laevis*, but expression is restricted to only neurons, muscle, and skin. To overcome this somewhat promiscuous expression in *Xenopus*, we specifically targeted pTAGUMGATA2, as well as a variant of this construct containing the CMV promoter (pTAGUMCMV), to dorsal blastomeres that are fated to become neural tissue. We find that this targeting strategy now restricts expression in muscle and skin to regions of the body axis that in many cases do not overlap with neural expression.

For *Xenopus* studies, targeted blastomeric injection yields embryos that should be useful for many neuroscience applications. For zebrafish studies, the pTAGUMGATA2 construct is neurospecific and simple; one-cell embryo injections yield embryos useful for exploring gene function in a neurospecific and cell autonomous context.

2. Time required

2.1. *Plasmid construction*

The plasmids pTAGUMGATA2 and pTAGUMCMV can be obtained from the corresponding author. Gene insertion into the backbone of these plasmids takes 3 days including screening for the insertion and preparing a DNA minipreparation of the plasmid.

2.2. *Zebrafish*

Injection of zebrafish embryos: injection requires 1 to 3 h. Growth of embryos: embryos are allowed to develop to 2 days p.f. when EGFP expression is maximal and constant. Immunohistochemical amplification of EGFP: to boost EGFP signals, fixed embryos are stained with an anti-GFP antibody. Immunohistochemical staining requires 2 days.

2.3. *X. laevis*

X. laevis females must be primed 1–2 weeks prior to the injection day and induced to ovulate the night before microinjection. Preparation of buffers used for *Xenopus* incubations and microinjection takes 2–4 h. Ovulation occurs 8–12 h post-injection of HCG. Isolating testes from males takes 30–45 min. From fertilization to the beginning of injection takes 30–45 min during which eggs must be dejellied. EGFP expression can be initially detected several hours after the onset of transcription at the mid-blastula transition (~8 h p.f.) but is optimally viewed after 48 h.

3. Detailed procedure

3.1. *Plasmid constructs*

The pTAGUMGATA2 plasmid was constructed by cloning EGFP sequences into the *Eco*RI/*Not*I sites of pSP64T (Melton et al., 1984). The resulting plasmid is called pEGFPT-N3. The GATA2 promoter sequence of nsP5-GM2 (Meng et al., 1997) was amplified by PCR using primers containing *Hin*dIII sites and inserted into the *Hin*dIII site of pEGFPT-N3 to create the plasmid pEGFPTns-N3. The SV40 early polyA signal from pEGFP-N1 (Clonetech Inc., Palo Alto, CA, USA) was amplified by PCR with *Not*I and *Xba*I sites in the primers. This fragment was used to replace the *Not*I/*Xba*I fragment of pEGFPTns-N3 containing the polyA and polyC stretches. The resulting plasmid is called pTAGUMGATA2 (Fig. 1A). pTAGUMCMV was constructed by removing GATA2 promoter and enhancer sequences from pTAGUMGATA2 and replacing them with CMV promoter sequences from pEGFP-N1. CMV promoter sequences were amplified by PCR using primers with *Hin*dIII sites.

3.2. *Zebrafish embryo injections*

Linear DNA for injections was generated by PCR using primers to vector sequences flanking the GATA2 promoter and SV40