Single-cell detection of microRNAs in developing vertebrate embryos after acute administration of a dual fluorescence reporter/sensor plasmid

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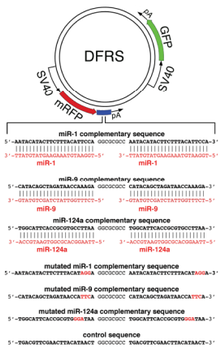
D.D.P.T. and F.C.: equal contribution

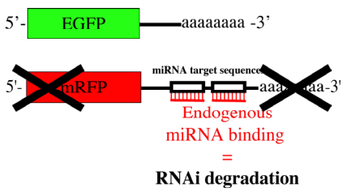
1) Introduction

MicroRNAs (miRNAs) are a class of 20/25-long nucleotides expressed in many organisms, from plants to mammals, which regulate the translation and stability of target mRNAs. MiRNAs are emerging as major players in the control of development and of several physiological and pathological processes. A crucial step towards a better understanding of miRNA function is the establishment of systems that allow their detection *in vitro* and *in vivo*. Reporter genes fused to a sequence complementary to the candidate miRNA, leading to the RNAi-mediated degradation of the reporter transcript, have been used as a system to detect miRNAs in living transgenic flies and mice (1,2). The major limitation of this technique is that it relies on the generation of transgenic animals making it infeasible to perform comprehensive screenings for miRNAs in these species. The topical release of nucleic acids in the developing mouse embryo, followed by directional electroporation, is a very efficient means for achieving either ectopic expression (3) or acute silencing (4) of a desired gene in mouse embryos developing either *in utero* or in culture. We decided to combine the system of *in utero* electroporation with a sensor-based approach in order to detect miRNA expression in specific tissues of developing mouse embryos.

2) The dual fluorescence reporter/sensor (DFRS) plasmid



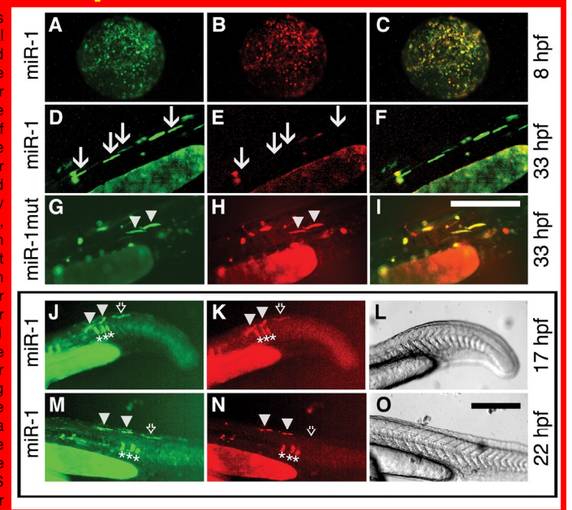
Top: Schematic map of the DFRS plasmid. Note the identical SV40 promoters driving GFP (green) and mRFP (red). Blue, tandem cassette–containing modified Unc54 3' UTR; pA, polyadenylation sites. **Bottom:** miRNA sensor sequences (black) and the corresponding miRNA sequences (red); mutated nucleotides in the sensor sequences are in shown in red.



The "sensor principle" is based on the RNAi-mediated degradation of the "sensor" (mRFP) transcript triggered by the presence in the cells of the miRNA of interest.

3) *In vivo* visualization of miR-1 dynamics during skeletal muscle development in zebrafish

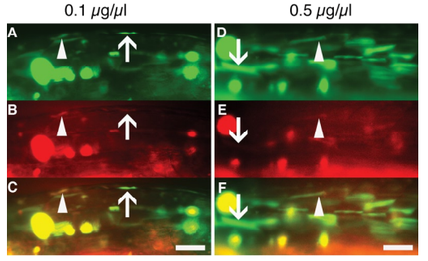
We tested a DFRS plasmid for miR-1, which has been shown to be expressed in developing skeletal muscle of zebrafish (5). The DFRS miR-1 plasmid was injected into one blastomere of 2–8–cell stage embryos, and embryos were allowed to develop for up to 9 days resulting in mosaic transgene expression. After 8 hpf (approximately 75-80% of epiboly), i.e. before the onset of skeletal muscle development, both GFP-reporter and mRFP-sensor fluorescence were observed, (A-C). This indicated that miR-1 was not yet expressed at this early stage of zebrafish development. At a later stage, i.e. after 33 hpf, when skeletal muscle has been formed, most of the GFP-expressing fibers did not show mRFP-sensor fluorescence (D-F, arrows). In contrast both GFP-reporter and mRFP-sensor fluorescence were observed in muscle fibers after blastomere injection of the mutated DFRS miR-1 plasmid 33 hpf (G-I, triangles). We therefore conclude that the lack of mRFP-sensor fluorescence in DFRS miR-1 plasmid–expressing muscle fibers at 33 hpf and thereafter indicated the presence of active miR-1. To explore whether a DFRS plasmid can be used to reveal the appearance of a miRNA in a given cell, we compared, after blastomere injection of DFRS miR-1 plasmid, the intensity of mRFP-sensor fluorescence in the same muscle fibers at two time points during earlier stages of skeletal muscle development, 17 hpf and 22 hpf. Remarkably, some muscle fibers showing both GFP and mRFP fluorescence at 17 hpf (J, K, open arrows) completely lost mRFP-sensor (N, open arrow), but not GFP-reporter (M, open arrow), fluorescence just 5 h later, and other muscle fibers that still showed mRFP-sensor fluorescence at 22 hpf (N, triangles) had lost it at 43 hpf (data not shown). In contrast, cells lacking miR-1 expression, such as those of the notochord (5), maintained both GFP-reporter and mRFP-sensor expression throughout (J, K, M, N, asterisks, and data not shown). These observations show that the DFRS miR-1 plasmid can be used to visualize the appearance of miR-1 activity in skeletal muscle during zebrafish development, which implies that the half-life of mRFP under the present conditions was sufficiently short to not obscure the appearance of the miRNA activity.



DFRS miR-1 plasmid (A-F, J-O) or mutated DFRS miR-1 plasmid (G-I) was injected into single blastomere of 2–4–cell stage (A-I) or 8-cell stage (J-O) zebrafish embryos, followed by analysis of GFP (A, D, G, J, M) and mRFP (B, E, H, K, N) fluorescence in cells after 8 h (A-C), 17 h (J-L), 22 h (M-O) and 33 h (D-I) post-fertilization (hpf); merge (C, F, I). Note that (J-L) and (M-O) are the same embryo 5 h apart; (L, O) bright field. Triangles indicate muscle fibers containing both GFP-reporter and mRFP-sensor. Arrows in (D, E) indicate muscle fibers showing GFP-reporter but no longer mRFP-sensor fluorescence. Open arrows in (J, K, M, N) indicate a muscle fiber that shows both GFP-reporter and mRFP-sensor fluorescence 17 hpf (J, K) but has lost the mRFP fluorescence 5 h later (N). Asterisks in (J, K, M, N) indicate notochord cells that maintain both green and red fluorescence. Fluorescent dots in panels M and N that are outside of the embryo presumably are autofluorescent methyl-cellulose crystals in the mounting medium used for live imaging. (D-O) Orientation of embryos: caudal, right; ventral, down. Scale bars = 250 µm; A-I and J-O, respectively are the same magnification.

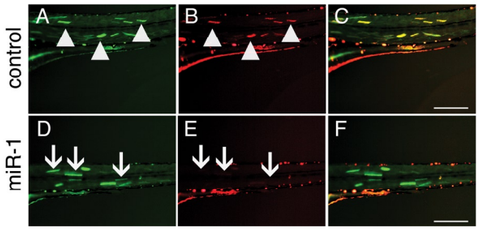
4) The silencing of the sensor is not affected by DFRS plasmid concentration and has a long-lasting stability of expression *in vivo*

miRNA detection is not impaired by variation in DFRS plasmid copy number.



DFRS miR-1 plasmid was injected into single blastomeres of 2-4–cell stage zebrafish embryos at a concentration of 0.1 µg/µl (A-C) or 0.5 µg/µl (D-F), followed by analysis of GFP (A, D) and mRFP (B, E) fluorescence in muscle fibers at 22 hpf; merge (C, F). Arrows indicate muscle fibers showing high-level GFP-reporter expression but no longer mRFP fluorescence; arrowheads indicate muscle fibers showing low-level GFP-reporter expression and some remaining mRFP fluorescence. Orientation of embryos: caudal, right; ventral, down. Scale bars = 70 µm.

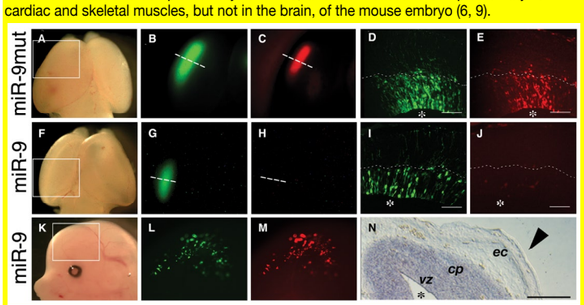
Detection of miR-1 expression in zebrafish muscle fibers after 9 days from injection.



DFRS control (A-C) or miR-1 (D-F) plasmid was injected into single blastomeres of 2-4–cell stage zebrafish embryos, followed by analysis of GFP (A, D) and mRFP (B, E) fluorescence in muscle fibers after 9 days post-fertilization; merge (C, F). Triangles indicate muscle fibers containing both GFP and mRFP. Arrows indicate muscle fibers showing GFP but no longer mRFP fluorescence. Orientation of embryos: caudal, right; ventral, down. Scale bars = 250 µm.

5) Detection of miR-9 in single neuroepithelial cells of mouse embryos after acute tissue targeting of DFRS plasmids

Given that Northern blot and microarray analyses and cDNA cloning have previously shown miR-9 to be expressed in the developing and adult rodent brain (6-8), we decided to use a DFRS miR-9 plasmid to detect this miRNA in the developing brain. As controls, we used (i) a DFRS miR-9 plasmid in which three nucleotides in each miR-9 complementary sequence were mutated in order to prevent miR-9 binding, (ii) a DFRS control plasmid the tandem cassette of which was not complementary to any known mouse miRNA, and (iii) a DFRS plasmid that contained, instead of the tandem cassette complementary to miR-9, a tandem cassette complementary to miR-1, which is known to be expressed in the developing cardiac and skeletal muscles, but not in the brain, of the mouse embryo (6, 9).



DFRS miR-9 plasmid (F-M) or mutated DFRS miR-9 plasmid (A-E) was injected into the lumen of the telencephalic vesicles (A-J) or administered in proximity of the ectoderm (K-M) of E13 mouse embryos in utero followed by electroporation into the left telencephalic neuroepithelium and left cranial ectoderm, respectively. After 24 h of *in utero* development, dissected brains (A-C and F-H) or whole embryos (K-M) were analyzed in the targeted region (boxes in A, F and K) for GFP (B, G and L) and mRFP (C, H and M) expression. Brains were then fixed and cryosections of the region of the neural tube wall showing GFP expression (B, G, dashed lines) were examined for the presence of GFP-reporter (D, I) and mRFP-sensor (E, J) in individual cells. Note the absence of mRFP-sensor fluorescence in the brain (H) and neuroepithelial cells (J) electroporated with the DFRS miR-9 plasmid, in contrast to its presence in the ectoderm electroporated with the DFRS miR-9 plasmid (M) and in the brain (C) and neuroepithelial cells (E) electroporated with the mutated DFRS miR-9 plasmid. (N) *In situ* hybridization using an LNA probe for miR-9 on coronal cryosections of the E14 mouse head. Note the absence of miR-9 in the ectoderm (N, ec arrowhead) and its presence in both the neuronal progenitors of the ventricular zone (N, vz) and postmitotic neurons in the cortical plate (N, cp) of the telencephalon. Asterisks indicate the ventricular lumen. Scale bars = 50 µm (D, E, I, J) and 100 µm (N).

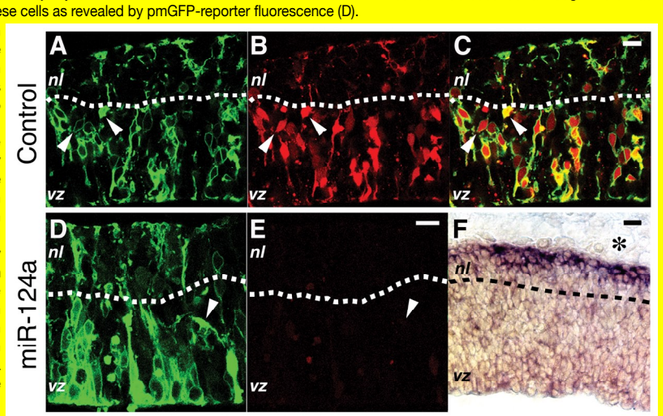
The DFRS plasmids were injected into the lumen of the telencephalic vesicles of embryonic day 13 (E13) mouse embryos *in utero* and electroporated into the left telencephalic neuroepithelium (A-J). After *in utero* development for 24 h, analysis of GFP-reporter and mRFP-sensor fluorescence showed that in the case of the mutated DFRS miR-9 plasmid, both fluorescent proteins were expressed in the E14 telencephalon (B, C), being colocalized in the same neuroepithelial cells (D, E). Similar results were obtained with the DFRS control plasmid or the DFRS miR-1 plasmid (data not shown), the latter finding being consistent with the lack of miR-1 expression in the developing brain (6, 7). In contrast, in the case of the DFRS miR-9 plasmid, only GFP-reporter (G, I), but not mRFP-sensor (H, J), fluorescence was detected, indicating the presence of this miRNA in E14 telencephalic neuroepithelial cells.

To corroborate the presence of miR-9 in the neuroepithelium, we performed *in situ* hybridization with an LNA probe (5) on cryosections through the head of E14 mice (N), which yield superior cellular resolution as compared to whole-mount preparations. Indeed, miR-9 was found to be abundantly expressed in the dorsal telencephalon of E14 mouse embryos (N), being present in both the neuronal progenitors in the ventricular zone (N, vz), i.e. neuroepithelial cells, and the postmitotic neurons in the cortical plate (N, cp), but being absent in the developing ectoderm (N, ec, arrowhead). As a further control, when the DFRS miR-9 plasmid was electroporated into the E13 ectoderm (rather than brain) followed by 24 h *in utero* development (K-M), both GFP-reporter and mRFP-sensor fluorescence were observed, being colocalized in the same cells (L, M). This showed that the DFRS miR-9 plasmid was functional in terms of GFP-reporter plus mRFP-sensor expression, and demonstrated the absence of miR-9 in the E14 ectoderm, consistent with the results of *in situ* hybridization (N, ec, arrowhead).

6) miR-124a is expressed not only in neurons but also in neuronal progenitors

We extended the DFRS approach to another miRNA, miR-124a, which is thought to be expressed specifically in neurons (8). During the development of the mouse central nervous system, newborn neurons prior to their migration to the neuronal layers co-exist with the neuronal progenitors in the ventricular zone. We therefore modified the DFRS plasmid by adding a plasma membrane localization signal to the GFP-reporter (pmGFP) in order to distinguish newborn neurons in the ventricular zone from neuroepithelial cells by outlining the distinct shape of these cell types. Indeed, when the DFRS control plasmid was electroporated into the E10 mouse telencephalic neuroepithelium *ex utero* followed by 24 h of whole-embryo culture, both the neuroepithelial cells in the ventricular zone (A-C, vz) and the neurons born in the ventricular zone (identified by the non-radial orientation of their cell body; A-C, vz arrowheads) and present in the neuronal layer (A-C, nl) showed pmGFP-reporter and mRFP-sensor expression. Surprisingly, however, upon electroporation of the DFRS miR-124a plasmid, essentially all cells, i.e. not only the neurons born in the ventricular zone (E, vz arrowhead) and present in the neuronal layer (E, nl) but also the neuroepithelial cells (E, vz), which constitute the vast majority of the cells in the ventricular zone, lacked mRFP-sensor fluorescence, although there was robust expression of the DFRS miR-124a plasmid in these cells as revealed by pmGFP-reporter fluorescence (D).

The same results were obtained upon electroporation of the DFRS miR-124a plasmid into the E13 mouse telencephalic neuroepithelium followed by 24 h of in utero development, and mutation of three nucleotides of each miR-124a target sequence was sufficient to prevent mRFP-sensor silencing (data not shown). These observations indicate that, contrary to the prevailing notion (10), miR-124a is expressed not only in postmitotic neurons but also in their progenitors, the neuroepithelial cells. These unexpected results on miR-124a were corroborated by *in situ* hybridization with a miR-124a LNA probe (8) on cryosections of E11 mouse brain, which confirmed the presence of a low level of miR-124a in the ventricular zone (F, vz) (in addition to the known massive expression in the neuronal layer; F, nl). The low-level *in situ* hybridization signal in the ventricular zone was specific as no such signal was detected in the ectoderm (F, asterisk), which is known to lack miR-124a expression. Thus, on a more general note, our findings demonstrate that the acute administration of a DFRS plasmid is a valid approach to gain novel information about the expression of a given miRNA.



(A-E) DFRS control (A-C) or miR-124a (D-E) plasmid was electroporated into the E10 mouse telencephalic neuroepithelium followed by 24 h of whole-embryo culture development, and cryosections were analyzed by confocal microscopy. Note the plasma membrane-localized GFP-reporter (A) and mRFP-sensor (B) fluorescence in neuroepithelial cells in the ventricular zone (vz), and in neurons in the ventricular zone (vz, arrowheads) and in the neuronal layer (nl) upon administration of DFRS control plasmid, but only pmGFP-reporter (D) and *not* mRFP-sensor (E) fluorescence in these cells upon administration of the DFRS miR-124a plasmid. (F) *In situ* hybridization using an LNA probe for miR-124a on a cryosection of E11 mouse telencephalon. Note the strong staining for miR-124a in the neuronal layer (nl) and the weak staining in the ventricular zone (vz). Dashed lines indicate the boundary between ventricular zone and neuronal layer. Scale bars = 10 µm.

Conclusions and perspectives

The present experimental approach of acutely administering a DFRS plasmid for a specific miRNA offers a convenient method to detect these important posttranscriptional regulators with single-cell resolution and to monitor their dynamics *in vivo*. Our approach, which presumably is applicable to a wide variety of species (including those for which transgenic lines has not been established), circumvents the need to generate transgenic organisms, which is much more labour-intensive for mice (2) than for flies (1). Moreover, the topical administration of DFRS plasmids, followed by their directed electroporation (3, 4), provides a simple approach to study a specific miRNA in the tissue and cell lineage of interest.

The mechanism of action of our system is based on RNAi, which is an efficient process, mediating the degradation of the sensor mRNA. We have shown *in vivo* that very low levels of miR-124a are enough to completely degrade high levels of sensor transcript, thus allowing the detection of low amounts of miRNAs. Moreover, RNAi is sequence-specific and we have shown that mutations of three nucleotides in the core sequence of a miRNA target can prevent the sensor mRNA degradation. We can therefore assume that our system is more sensitive and specific than microarrays.

Our DFRS plasmid can be easily used for the preparation of libraries, thereby allowing fast read-out of either *in vivo* or *in vitro* screenings.

References

1) Brennecke, J., Hipfner, D.R., Stark, A., Russell, R.B. & Cohen, S.M. Cell 113, 25-36 (2003).
2) Mansfield, J.H. et al. Nat Genet 36, 1079-1083 (2004).
3) Osumi, N. & Inoue, T. Methods 24, 35-42. (2001).
4) Calegari, F., Haubensak, W., Yang, D., Huttner, W.B. & Buchholz, Proc Natl Acad Sci U S A 99, 14236-14240 (2002).
5) Wienholds, E. et al. Science (2005).
6) Lagos-Quintana, M., R. Rauhut, A. Yalcin, J. Meyer, W. Lendeckel and T. Tuschl, Curr Biol 12:735-739 (2002).
7) Krichevsky, A.M., K.S. King, C.P. Donahue, K. Khrapko and K.S. Kosik, RNA 9:1274-1281 (2003).
8) Miska, E.A., et al. Genome Biol 5:R68 (2004).
9) Zhao, Y., Samal, E. & Srivastava, D. Nature 436, 214-220 (2005).
10) Smirnova, L., A. Grafe, A. Seiler, S. Schumacher, R. Nitsch and F.G. Wulczyn, Eur J Neurosci 21:1469-1477 (2005).

De Pietri Tonelli D., Calegari F., Fei J.F., Nomura T., Osumi N., Heisenberg C.P. and W.B. Huttner. **Biotechniques** (2006) *in press*.