

Supplemental Material

Title: Imaging Cerebral Gene Transcripts in Live Animals

Background Aspects of two disciplines will be employed in the studies to properly visualize gene expression in live animal brains: 1) target-specific oligodeoxynucleotides (ODN) with sequences complementary to c-fos mRNA, or β -actin mRNA, and 2) MR contrast conjugates that can be introduced to brain tissue. The basis of ODN delivery and uptake has been demonstrated in at least two reports (Liu et al. 1994; Cui et al. 1999), and independently repeated by at least one group of investigators (Zhang et al. 1999). Here we show the distribution of ODN in the first day after delivery (unpublished observations).

Convection Delivery using Intracerebroventricular Infusion: Because quenching effect in SPION-linked FITC-sODN, we delivered phosphorothioate-modified and digoxigenin-labeled ODN with antisense sequence for c-fos (sODN-cfos-dig, at 18 nmoles

in 10 μ l of 25 mM sodium citrate buffer [pH 8.0] with 0.1 μ g/ml lipofectin), via intracerebroventricular infusion to the left ventricular space (Liu et al. 1994; Cui et al. 1999). The distribution was allowed for one day through ventricular circulation. We have shown in previous publication that the sODN attaches itself to the ventricular walls and then gains entry to neurons in which cerebral c-fos mRNA is expressed. The presence of sODN–dig was detected using fluorescence isothiocyanate (FITC) labeled immunoglobulin (IgG) against dig. The distribution of sODN in one day is approximately 15 mm because we also observe the presence of sODN-dig-FITC in neurons of the cerebellum and the olfactory bulb (Figl S1A-B). The FITC is not autofluorescence of the tissue because deletion assay (minus FITC-IgG) shows no fluorescence (Fig S1C).

Figure S1A shows uptake and distribution of FITC-sODN in the nucleic of the cerebellar folium, including Purkinji neurons (panel S1A, arrow). The ICV route of delivery is similar to that of convection route.

SPION and FITC-sODN Linkage: The superparamagnetic iron oxide nanoparticles (SPION) used in this manuscript cannot be separated from solution using magnetic bars.

We have developed a gel electrophoresis method to examine conjugation of SPION to phosphorothioate-modified oligodeoxynucleotides (sODN) that are labeled with fluorescein isothiocyanate (FITC) for tracing and with biotin for conjugation to NeutrAvidin (NA).

Methods: Activated SPION with NA (SPION-NA) was mixed with various amounts of FITC-sODN-biotin, incubated at room temperature for 30 minutes, and applied to a 0.8% agarose gel. We photographed the gel as noted in the Materials and Methods section of the text.

Results: Figure S2 shows binding of increasing doses of FITC-sODN-biotin to 270 pmol freshly prepared SPION-NA (before purification with a Centricon Plus-100 filter). No fluorescence in the lane with unconjugated SPION-NA (lane marked 0) indicated there was no spill over of FITC-sODN-biotin during gel loading. There were two visible bands for lanes loaded FITC-sODN-biotin: one at 8 cm and the other at 6 cm with a smear. The

8-cm band is unconjugated FITC-sODN. There was no change in fluorescence among samples with 150, 200, 250 and 300 pmol FITC-sODN (lanes with 1.5 – 3 μ l), but an increase in fluorescent intensity did appear in samples between 50 and 150 pmol (and above). A small amount of FITC was seen at 8 cm in those lanes with samples of 150–300 pmol, but intensity increased in samples with 600 or more pmol of FITC-sODN (long arrow, Fig S2). The saturation dose was less than 600 pmol sODN to 270 pmol SPION-NA. At doses higher than the saturation concentration, there is a gradual increase in signal intensity and spill-over to surrounding gel space. Because in this case SPION-NA had not yet undergone purification, no binding site is calculated.

No fluorescence could be seen at 1 cm, near the well (horizontal arrows), in each lane where we generally detected SPION. After the first photoimaging (Fig S2), the gel was manually transferred, with gloved hands, from the UV light box to the gel tray for further electrophoresis. After electrophoresis at 1500 volt-hours (Fig S3), fluorescent signal was present, though weak, and appeared as a smear in each lane except that with only SPION-NA. Several fluorescent dots were observed in the gels (Fig S3); these dots

originate from lengthy exposure to UV light and most likely form when the gel is handled with powdered latex gloves. Fig S3 shows faint fluorescence located within 2 cm of the well in lanes where doses above the saturation dose of FITC-ODN (> 300 pmoles) were used. The presence of excess NeutrAvidin in the unpurified SPION-NA may account for faint binding, SPION-NA was filtered dialyzed using Centricon Plus-100 against 20x volume of sodium citrate (25 mM, pH=8) to remove NA (65 Kd).

Binding of FITC-sODN-biotin to purified SPION-NA was again tested in samples with equal molar ratios of sODN and SPION-NA (Fig S4, lanes 1–8). When the gel, without additional fluorescent dye for DNA, was illuminated with UV light for imaging at an exposure time of 8 seconds, the bright bands that were present before filtration were no longer observed in samples with SPION-NA and FITC-sODN conjugates (Fig S4).

These results suggest that NA is removed from freshly prepared SPION-NA and allows conjugation of FITC-sODN to SPION-NA; however, fluorescence is observed as a smear starting from the well in each lane. The signal weakness necessitated the rather lengthy photography exposure time of 8 seconds.

There were 2 fluorescent signals in Fig S4: one at 1 cm from the well and the other at 11 cm. Free and unconjugated FITC-sODN-biotin migrated to 11 cm and fluorescent intensity spilled over into surrounding spaces (lane 1, long arrow, Fig S4). A few bright dots, believed to have resulted from gel handling, were also present in the gel (lane 5, Fig S3). Because 120 pmole of purified SPION-NA binds to 120 pmole FITC-sODN-biotin with as small amount of FITC-sODN left over at 11 cm (lane 5, Fig S4), we concluded saturation of SPION-NA had occurred at one to one molar ratio or one biotin per SPION-NA.

When SPION-NA and FITC-sODN-biotin were mixed before loading, the majority of fluorescence signal sifted from 11 cm to 1 cm, with most of the dye having traveled only 1 cm at a distance similar to SPION in lanes 4& 5. When one of the two components was not included in the mixing, no gel shift was present (lanes 1, 6 7 & 8). The result indicates there is a band shift typical of DNA-protein binding, or conjugation of FITC-sODN and SPION-NA via biotin on sODN and NA on SPION.

Discussion and Conclusion We described a new and direct assay to demonstrate conjugation of SPION-NA to DNA using gel-shift assay. This assay is based on migration retardation of a labeled DNA after binding to protein. Here we mixed FITC and biotin labeled oligo DNA to NeutrAvidin (NA) labeled SPION. By conjugation between NA and biotin, we demonstrated a gel shift from FITC-sODN-biotin SPION-sODN-FITC.

When SPION-NA and starting material could not be purified with a conventional magnetic column, we purified SPION-NA from NA with centrifugal separation using Centricon Plus-100 filters. The disappearance of NA binding after separation allows detection of SPION-NA binding with a tail smear. By removing excess NA, we also calculated the number of binding sites for biotin in purified SPION-NA. We do not know the origins of the smear, but we believe it represents the size distribution of SPION-NA. Because of the size of the sODN (less than 9 KD), binding of smaller molecular weights to SPION-NA would not appear as a sharp band as with plasmid DNA of several kilo base pairs.

References

- Cui, J. K., C. Y. Hsu, et al. (1999). "Suppression of postischemic hippocampal nerve growth factor expression by a c-fos antisense oligodeoxynucleotide." J Neurosci **19**(4): 1335-44.
- Liu, P. K., A. Salminen, et al. (1994). "Suppression of ischemia-induced fos expression and AP-1 activity by an antisense oligodeoxynucleotide to c-fos mRNA." Ann Neurol **36**(4): 566-76.
- Zhang, Y., M. A. Widmayer, et al. (1999). "Suppression of post-ischemic-induced fos protein expression by an antisense oligonucleotide to c-fos mRNA leads to increased tissue damage." Brain Res **832**(1-2): 112-7.
- Liu, CH, Huang S, Kim YR, Rosen BR and Liu PK (2006) Forebrain Ischemia-reperfusion Simulating Cardiac Arrest in Mice induces Edema and DNA Fragmentation in the Brain (J. Mol Imaging, in press).