**TECHNICAL NOTE**

**N-Ethylmaleimide (NEM) Can Significantly Improve In Situ Hybridization Results Using $^{35}$S-labeled Oligodeoxynucleotide or Complementary RNA Probes**

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**SUMMARY** We predicted that a significant source of background labeling after in situ hybridization (ISH) using $^{35}$S-labeled probes is attributable to a chemical reaction between the phosphorothioate moiety of the probe $\text{[O}_3\text{P}^-\text{S]}$ and disulfides in tissue. These covalent bonds would immobilize probe in the tissue, thereby increasing background labeling. On the basis of this view, we have explored the use of N-ethylmaleimide (NEM) to irreversibly alkylate the phosphorothioate moiety of the probe and/or to alkylate free sulfhydryls in tissue to block the formation of disulfides as a method of reducing background labeling. We report that NEM can significantly decrease background labeling of $^{35}$S-labeled oligodeoxynucleotide or cRNA probes but does not affect specific labeling. We conclude that the use of NEM in ISH protocols, as outlined here, may be an additional element researchers may consider to improve the signal-to-noise ratio.

**KEY WORDS** in situ hybridization
N-ethylmaleimide
$^{35}$S-labeled probes
oligodeoxynucleotide
cRNA

**THERE ARE TWO SOURCES** of nonspecific labeling (background) of tissue after in situ mRNA hybridization (ISH): cross-hybridization to related sequences, and probe binding to non-RNA components in the tissue. Cross-hybridization can be limited or eliminated by a combination of careful probe design and manipulation of hybridization and wash conditions (Altar et al. 1989). In contrast, probe binding to non-RNA components is governed by chemical interactions, which cannot be addressed by alteration of stringency. Therefore, optimizing ISH protocols represents a balance between conditions required for selective hybridization and conditions that limit probe binding to non-RNA components. However, this balance can be difficult to achieve when ISH is performed for target mRNAs of low abundance, including many important mRNAs expressed in brain, because the two most obvious procedural modifications—increasing the specific activity of the $^{35}$S-labeled probe and increasing the duration of exposure to photographic film or emulsion—can lead to unacceptably high background (e.g., Bradley et al. 1992). Therefore, a variety of approaches must be available to investigators as they optimize ISH protocols for specific target mRNAs.

Considering that radioactive probes used in ISH protocols are most often labeled with $[^{35}\text{S}]\text{dATP}$ or $[^{35}\text{S}]\text{-UTP}$, in which the $^{35}$S replaces oxygen on the $\alpha$-phosphate, forming a phosphorothioate $[\text{O}_3\text{P}^-\text{S}]$, we hypothesized that one source of background in ISH protocols using $^{35}$S is due to oxidation of free sulfhydryls in tissue, with subsequent interaction of the resulting disulfides with the phosphorothioate moiety of the probe. This hypothesis is based on work demonstrating that free phosphorothioate can interact with sulfides by a nucleophilic scission reaction, liberating a thiol and producing an adduct $[-\text{O}_3\text{PS-SR}-]$ (where SR is the sulfur of another molecule such as cysteine) (Neumann and Smith 1967). The use of high...
concentrations of the reducing agent dithiothreitol (DTT; 10–100 mM) in the hybridization buffer to reduce background appears to support this hypothesis. If DTT reduces endogenous disulfide bridges and limits the formation of new ones during the hybridization, there would be less disulfide to react with the phosphorothioate of the probe.

The use of DTT to control background labeling in ISH protocols is compromised, however, because it is a relatively slow reducing agent and can be oxidized by molecular oxygen (Lees et al. 1991; Singh and Whitesides 1991). This can be visualized by carrying out the hybridization for different durations. Background will increase with increasing duration of hybridization (personal observations). Therefore, we have considered the possibility that background labeling in ISH protocols could be reduced by limiting the interaction of the probe with disulfides in tissue. Our reasoning was that irreversible alkylation of free sulfhydryls in tissue with N-ethylmaleimide (NEM) before hybridization would limit further disulfide formation in tissue and reduce background. In addition, alkylating the phosphorothioate moiety of the probe directly was that irreversible alkylation of free sulfhydryls in tissue with N-ethylmaleimide (NEM) before hybridization would limit further disulfide formation in tissue and reduce background. In addition, alkylating the phosphorothioate moiety of the probe directly may also block its ability to interact with disulfides. NEM forms a sulfur–carbon bond at room temperature and neutral pH that is irreversible under these conditions (Jocelyn 1972; Friedman 1973).

We tested the effects of NEM on characteristics of the ISH for the mRNA encoding thyrotropin-releasing hormone (TRH) in rat brain. We chose this particular RNA for our experiments because we have previously validated the use of both the oligodeoxynucleotide probe (Zoeller et al. 1993) and the cRNA probe (Zoeller et al. 1995), and because the distribution of TRH mRNA expression is quite well understood in rat brain (Segersten et al. 1987a; Sevarino and Primus 1993). In addition, we tested the potential interaction between the use of NEM and the use of DTT in the ISH protocol for oligodeoxynucleotide probes because, in principle, NEM may obviate the requirement for DTT. In addition, we tested the potential interaction between the use of NEM and RNAse A in the ISH protocol for cRNA probes because it is possible that RNAse treatment is removing probe immobilization by formation of disulfide interactions.

Materials and Methods

Experimental Design

Oligonucleotide Probe. In this experiment, we tested three variables on characteristics of the hybridization signal and background: NEM treatment of probe (NEM), NEM treatment of tissue (NEM), and use of DTT in the hybridization buffer. This resulted in 8 (2 × 2 × 2) different experimental groups (±DTT/±NEM/±NEM). Each group consisted of four sections; all sections were taken from the same PVN and all were included in the same hybridization assay. This full experiment was then repeated three times.

cRNA Probe. In this experiment, we tested the effect of NEM, NEM, and the use of RNAse A in the wash. This resulted in 8 (2 × 2 × 2) different experimental groups (±RNAseA/±NEM/±NEM). Each group consisted of four sections; all sections were taken from the same PVN and all were included in the same hybridization assay. This full experiment was then repeated three times. These treatments are described below.

Probe Preparation

The plasmid containing the full-length cDNA (1.2 kb) encoding rat TRH cloned into pSP64 was kindly provided by Dr. R.H. Goodman (Lechan et al. 1986b; Vollum Institute, Portland, OR). After amplification in E. coli (DH5a; Gibco/BRL, Gaithersburg, MD), the plasmid was separated from bacterial DNA using the Qiagen MidiPrep Kit (Chatsworth, CA), linearized with HindIII and transcribed with SP6 RNA polymerase in the presence of [35S]-UTP (New England Nuclear; Boston, MA) using reagents from the Riboprobe Core II transcription system (Promega; Madison, WI). The transcription was performed in the presence of 500 μM each of ATP, CTP, and GTP, 6 μM [35S]-UTP, and 6 μM UTP. The oligodeoxynucleotide was synthesized on an Applied Biosystems M dode 380B (South San Francisco, CA) DNA synthesizer and purified on an RPC column by the University of Missouri DNA Core Facility. This oligo contains 48 bases (GTC TTT TTC CTC CTC CCT TTT GCC TGG ATG CTG GCC TTT GTG GAT) complementary to bases 366–319 of the rat TRH mRNA (Lechan et al. 1986b), and exhibits significant identity only to mammalian TRH among DNA sequences in Genbank using the Basic Local Alignment Search Tool (BLAST) at the internet site www.ncbi.nlm.nih.gov. The oligodeoxynucleotide (5 pmol) was 3' end-labeled by incubating with 50 U terminal deoxynucleotidyl transferase (TdT; Boehringer Mannheim; Indianapolis, IN), 50 pmol of [35S]-dATP (New England Nuclear), 200 mM potassium cacodylate, 25 mM Tris-HCl, 0.25 mg/ml bovine serum albumin, and 1.5 mM CoCl2 (pH 6.6) for 15 min at 37°C. This procedure will transfer, on average, 10 [35S]-dAMP per oligomer.

Treatment of Probe with NEM

After the labeling reaction of either oligomer or cRNA, the probes were ethanol-precipitated in the presence of 0.4 M NaCl and the resulting pellet was redissolved in 100 μl T E (10 mM Tris, pH 7.4, 1 mM EDTA) (oligomer), or 100 μl 0.1% SDS (cRNA). This material was then divided into two equal aliquots and ethanol-precipitated once again. The resulting pellets were dried under vacuum and dissolved in 50 μl of 1 × SSC (onefold concentrated standard saline citrate = 0.15 M NaCl/0.015 M sodium citrate) or 1 × SSC/50 mM NEM (Sigma Chemical; St. Louis, MO). All probes were then heated to 37°C for 30 min and stored at 4°C (oligomer) or –80°C (cRNA). This procedure, in which the probe prepared in a single labeling reaction was divided into two aliquots and separately treated with NEM or NEM, ensured that differences in hybridization characteristics after ISH using these different probes are related only to the NEM treat-
ment. Treatment of cRNA probes with NEM required careful attention to controlling RNAses so that probe was not compromised during the 37°C incubation.

Tissue Preparation

Animals were treated in accordance with NIH guidelines as approved by the University of Massachusetts IACUC. Brain tissue was dissected fresh from rat (Sprague-Dawley, Charles River, Wilmington, MA) and immediately frozen in pulverized dry ice. Coronal sections were cut in a Reichert-Jung Frigocut 2800N cryostat (Leica; Deerfield, IL) at 12 μm through the hypothalamic paraventricular nucleus (PVN), and thaw-mounted onto cold twice gelatin-coated slides. After the sections had briefly air-dried, they were stored at −80°C until the hybridization.

In Situ Hybridization

Prehybridization. Our standard prehybridization procedure is identical for use with oligonucleotide and cRNA probes. This standard protocol is as follows. Sections are first removed from the −80°C freezer, warmed to room temperature, loaded into plastic Coplin jars that had been previously treated with diethylpyrrocarbonate (DEPC; Sigma), and autoclaved (Blumberg 1987). Sections are then fixed in 4% formaldehyde/phosphate-buffered saline (PBS; 0.15 M NaCl/1.0 mM KH2PO4/6.0 mM Na2HPO4) for 5 min, rinsed in PBS for 2 min, and soaked for 10 min in 0.25% acetic anhydride in 0.1 M triethanolamine hydrochloride/0.9% NaCl (pH 8.0). They are then rinsed in 2 × SSC, dehydrated through a graded series of ethanol (70% 1 min; 80% 1 min; 95% 2 min; and 100% 1 min), delipidated in chloroform for 5 min, rehydrated to 95% ethanol, and air-dried.

NEM Treatment of Tissue. We performed a number of preliminary experiments to determine how to incorporate NEM treatment of tissue into this prehybridization protocol. Our initial reasoning was that NEM treatment should be performed after tissue fixation so that endogenous RNAses activity would be reduced and tissue sections would remain affixed to the slides. In the present study, we included NEM treatment following the 1 × SSC rinse after the acetylation step because the diluent for NEM is 1 × SSC and tissues would already be equilibrated to this solution. Therefore, after this 1 × SSC rinse, sections were immersed for 20 min in either 50 mM NEM/1 × SSC or 1 × SSC. After an additional 1 × SSC rinse for 1 min, sections were then dehydrated, delipidated, and air-dried as described above.

Hybridization. After air-drying, the sections were placed in the bottom of a Nunc box and 50 μl of hybridization buffer containing 106 cpm probe was applied. The buffer was evenly distributed over the tissue by application of a parafilm coverslip, the lids were placed on the boxes with small receptacles of water placed inside to provide humidity, and tissues were incubated overnight at 45°C (cRNA probe) or 37°C (oligomer). The hybridization buffer used for the oligonucleotide probe contained 50% formamide, 4 × SSC, transfer RNA (250 μg/ml), sheared, single-stranded salmon sperm DNA (100 μg/ml), Denhardt’s solution (0.02% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone), 10% (w/v) dextran sulfate (molecular weight 500,000). For hybridizations with the TRH oligonucleotide, we tested the possibility that DTT (Fisher Scientific; Pittsburgh, PA) in the hybridization buffer would interact with NEM treatments of tissue and/or probe. Therefore, some tissues were hybridized in buffer containing 50 mM DTT and others were not. The hybridization buffer used for the cRNA probe was identical to that described above but contained 2 × SSC and 50 mM DTT.

Washing. After hybridization, the coverslips were floated off in 1 × SSC and washed four times for 15 min each in 1 × SSC on a rotary shaker at room temperature. Sections hybridized with the oligodeoxynucleotide probe were further washed four times for 15 min each in 50% formamide/2 × SSC at 40°C, followed by two 30-min washes in 1 × SSC. Sections hybridized with the cRNA probe were further washed as follows: twice for 10 min each in 50% formamide/2 × SSC at 52°C; twice in 2 × SSC for 3 min each; 30 min in 2 × SSC/100 μg/ml RNase A at 37°C; twice in 2 × SSC for 3 min each; twice in 50% formamide/2 × SSC at 52°C for 10 min each; and twice in 1 × SSC for 30 min each. Some cRNA-hybridized sections were not treated with RNase A but were soaked in 2 × SSC at 37°C for control purposes. After the washing procedure for either the oligodeoxynucleotide or cRNA probe, the sections were dehydrated through graded ethanol (1 min each in 70%, 80%, and 95%) and air-dried.

Autoradiography, Analysis, and Statistics

After the slides were air-dried, they were arranged in X-ray cassettes and apposed to film (Kodak Bio-Max; Rochester, NY) for 15 hr (TRH cRNA) or 3 days (TRH oligomer). Characteristics of the hybridization signal were analyzed on a Macintosh IIfx computer using the public domain NIH Image program (available through the NIH website—http://www.nih.gov). This system was interfaced with a Dage-MTI 72 series video camera equipped with a Nikon macro lens mounted onto a bellows system over a light box. 12C-standards were used to ensure that the ISH signal was on the linear portion of the response curve for the film. Signal characteristics were evaluated for each probe as follows. First, the average density (gray level, on a scale from 0 to 255) was measured over each PVN (signal). Second, the average density was measured over an area of the thalamus that does not express TRH mRNA (background) (Lechan et al. 1986a; Koller et al. 1987; Segersen et al. 1987a,1987b). Signal:noise ratio was then calculated as the ratio of density of the signal to that of background. Statistical analyses were performed on these density values. A three-way analysis of variance was performed on these measures, with main effects being DTT, NEMp, and NEMT for the TRH oligomer and RNaseA, NEMp, and NEMT for the TRH cRNA.

Results

Oligonucleotide Probe

The inclusion of DTT in the hybridization buffer was essential for controlling background, even in the presence of NEM (Figures 1 and 2). DTT, in the absence of NEM treatment, decreased background from 131.8 ±
8.12 (mean film density ± SEM) to 57.1 ± 2.1. This was highly statistically significant \( F(1,24) = 615.9; p << 0.001 \). In the absence of DTT, NEM treatment was without effect. In contrast, in the presence of DTT, NEM treatment of the probe \( F(1,12) = 75.52; p << 0.001 \) or tissue \( F(1,12) = 45.12; p << 0.001 \) significantly reduced background. The combination of NEM treatment of probe and tissue tended to reduce background further (compared to NEM treatment of probe or tissue) but this was not statistically significant. However, there was no significant effect of NEM on the intensity of the signal over the PVN, which accounted for a twofold increase in the signal:noise ratio in tissues treated with NEM and hybridized with a probe treated with NEM (Figure 2).

cRNA Probe

Treatment of tissue hybridized with a \(^{35}\)S-labeled cRNA probe with RNase A was essential for controlling background, even in the presence of NEM (Figures 3 and 4). RNase A produced a 3.5-fold reduction in background labeling \( F(1,24) = 222.9; p << 0.001 \). In the absence of RNase A treatment, NEM treatment of the cRNA probe produced a nearly twofold reduction in background labeling \( F(1,24) = 29.9; p = 0.0013 \). Although this was not as remarkable for tissue treated with RNase A, NEM treatment of tissue and probe did produce a small but significant increase in the signal:background ratio \( F(1,24) = 4.09; p < 0.05 \).

Discussion

The purpose of these experiments was to systematically evaluate whether irreversible alkylation of free sulfhydryls in tissue, or of the phosphorothioate moiety of the probe, could significantly improve the quality of in situ hybridization results. We report that NEM treatment of probe and/or of tissue significantly reduces the background labeling after ISH with \(^{35}\)S-labeled oligonucleotide or cRNA probes, thereby enhancing the signal:noise ratio. These results are consistent with
the hypothesis that a measurable proportion of background labeling after ISH with $^{35}$S-labeled probes is due to interaction of the phosphorothioate moiety of the probe with disulfides in tissue. These disulfides presumably are associated with proteins in general rather than with a specific class of functional proteins. Differences in abundance of disulfides among various tissues may explain why different tissues exhibit different levels of background labeling after hybridization with the same probe, and NEM treatment may be more useful in some tissues than in others.

NEM treatment improved signal characteristics after hybridization with both the oligodeoxynucleotide and cRNA probe for the same target RNA (TRH). The inclusion of DTT in the hybridization buffer for the oligonucleotide probe is clearly important in controlling background (Figures 1 and 2). The average gray level attributable to background in the absence of DTT in the hybridization buffer dropped nearly threefold in the presence of 50 mM DTT. NEM treatment produced an additional twofold reduction in background labeling. However, NEM did not appear to influence background labeling in the absence of DTT. This observation may indicate that tissue disulfides should be reduced to form free thiols before NEM treatment to achieve a greater effect of NEM.

NEM treatment also improved the signal:noise ratio after ISH with the $^{35}$S-labeled cRNA probe. However, there are two components of our results that warrant discussion. First, treatment with RNAse is essentially required to reduce background labeling to acceptable levels. Most if not all ISH protocols using cRNA probes incorporate RNAse treatment for this reason. This indicates that RNA probes interact with tissue in ways that are independent of hybridization events or of the chemistry of sulfur, and which are separate from single-stranded DNA probes. This interpretation is supported by the appearance of the background labeling of the cRNA probe in the absence of RNAse treatment (compare Figures 1A and 3A). Specifically, this background appears to be cellular, as indicated by a pattern of high background in cell-rich zones (e.g., cortical layers, hippocampus, caudate). In comparison, the pattern of background after ISH with the oligodeoxynucleotide probe appears to be unrelated to cell density, even in the absence of DTT. Second, we observed only a small but significant increase in the signal:noise ratio of NEM treatment in tissues treated with RNAse. It is likely that this minimal effect is due mainly to the low background already observed in these tissues (Figures 3 and 4). Although we predict that effects of NEM treatment on background labeling would be more robust if the sections were exposed to film for longer durations, the signal over the PVN would become saturated and the signal:noise ratio would appear to be diminished. Therefore, a different target RNA would have to be evaluated to test this hypothesis. The effect of NEM observed in tissues not treated with RNAse A may be more indicative of effects in situations where the tissues are exposed to film for much longer periods (i.e., where signal:noise ratio is lower). In this case, NEM treatment of the cRNA probe reduced background by nearly 50% (114.3 ± 9.3 vs 67.6 ± 4.6) and produced a doubling of the signal:noise ratio. This finding indicates that NEM may be useful in ISH protocols designed to study low-abundance mRNAs in which high specific activity cRNA are required, or where a high concentration of the probe is used to drive hybridization over a shorter time period, or where long durations against emulsions are required.

The practical value of these findings will depend on the specific applications various investigators may have. For example, the mRNA encoding arginine vasopressin expressed in magnocellular neurons of the rat hypothalamic paraventricular nucleus is extremely abundant, and we routinely detect this mRNA with a $^{35}$S-labeled oligodeoxynucleotide probe after 30–45-min exposure to X-ray film (Zoeller et al. 1990). In this case, we have no detectable background and we would not use additional methods, such as NEM treatment, to control background.

In contrast, mRNAs of intermediate to low abundance, such as those encoding TRH, corticotropin-releasing hormone (CRH), gastrin-releasing peptide (GRP), or vasoactive intestinal peptide (VIP), require
1 week or more against film (Albers et al. 1989; Zoeller et al. 1990; Dolan et al. 1992; Zoeller and Rudeen 1992), and additional methods of controlling background are clearly of value. Our present findings indicate that NEM treatment of the \(^{35}\)S-labeled probe and of the tissue can improve the quality of ISH results. NEM treatment of the probe appeared to be the more effective of the two procedures we evaluated. In our initial experiments, we tested for the optimal concentration of NEM (10–100 mM), duration of treatment (5–30 min), and sequence within the prehybridization protocol for treatment of tissue (data not shown). We also considered the possibility that NEM treatment of the tissue may be more effective after DTT treatment to reduce disulfide bonds first. Although this did not enhance the ability of NEM treatment of tissue to reduce background labeling, it is possible that procedures could be manipulated to enhance the effect of NEM treatment of tissue.

NEM treatment may be especially useful in situations in which multiple oligodeoxynucleotide probes

![Figure 3](image-url) Effect of RNase and NEM treatments on the signal after in situ hybridization for TRH mRNA in rat brain using a \(^{35}\)S-labeled cRNA probe. Each panel represents the autoradiographic film image of a coronal section through rat brain that was digitized using a DAGE Series 72 video camera, as described in the text. All images were captured under identical conditions so that visual comparison among panels is valid. Film density in Figure 4 refers to degree of darkness or increasing gray level in these panels. These images were then imported into Photoshop 4.0 for purposes of figure assembly without further image editing. Brain sections were hybridized under the following conditions: absence of RNase in the wash or NEM treatment (A); absence of RNase in the wash, NEM treatment of tissue (B); absence of RNase in the wash, NEM treatment of probe (C); RNase treatment plus NEM treatment of probe and tissue (D). Specific labeling is best visualized in D, in which the hypothalamic paraventricular nucleus (PVN) and reticular nucleus of the thalamus (RNT) are both labeled. Scattered cells ventral and lateral to the PVN are also labeled with the TRH probe, but these were not analyzed in this study. Note the cellular appearance of the background (compare D and A). We are unsure of the cause of the variation in background apparent in the absence of RNase treatment (A–C). This was common. Cpu, caudate putamen; Cx, cortex.

![Figure 4](image-url) Quantitation of image characteristics after in situ hybridization for TRH using a \(^{35}\)S-labeled cRNA probe. Bars represent mean ± SEM of film density over the PVN (signal) and thalamus (background) for each treatment group, as described in the text. Higher film density refers to increasing darkness of the film as visualized in Figure 3. The eight treatment groups are: left panel, no RNase treatment in the wash; right panel, 100 mM RNase A in the post-hybridization wash. NEMP, NEM treatment of probe; NEMT, NEM treatment of tissue. Note that the difference in density between signal (PVN) and background is much greater with a cRNA probe than for the oligonucleotide probe.
are used to detect the distribution of very low-abundance mRNAs, such as the adrenergic receptors (Nicholas et al. 1993; Pieribone et al. 1994) or thyroid hormone receptors (Bradley et al. 1989). Considering that NEM treatment of neither probe nor tissue affected the intensity of the signal, there appears to be little risk in evaluating this method for a particular application.

There are a variety of procedures one can use to optimize the signal/noise ratio in protocols for in situ hybridization. We report here the use of NEM to control background associated with chemical reactions involving 35S-labeled probes. This method may be of value to investigators using ISH in tissues that produce high levels of background or where the target mRNA is very low in abundance. NEM treatment may also reduce the cost of using other strategies, such as very high concentrations of DTT and/or the use of 33p.

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