

Distributed Radiation Monitoring Via a Secure Wireless Sensor Platform

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Abstract

Sensors have long been used to monitor radiation emanating from nuclear materials throughout their life cycle. However, many of these systems have relied on area monitoring techniques which are designed to detect background changes or hard-wired installations focused on item monitoring that can be costly to install [4]. Current revolutions in low-power wireless devices could be used to improve the feasibility of utilizing radiation monitoring for nuclear safeguards that require item monitoring. Wireless sensors provide easier setup, lower install cost, and more flexibility in dynamically changing environments.

The requirements of a wireless sensor platform for use in distributed radiation monitoring applications were investigated. This paper will discuss a proposed platform to meet the requirements of nuclear safeguards, and attempt to estimate the power consumption and battery life of this device. This extends previous work done on developing a similar Ethernet connected platform [5]. At the time of this paper, a complete design and final testing of the platform has not yet been accomplished, and is planned as future work.

Introduction

Commercial off-the Shelf (COTS) technology has always had a significant influence on the device communication techniques deployed in nuclear safeguards. Advances in outside markets are frequently adapted by nuclear safeguards efforts to provide more cost-effective and more secure monitoring methods [1]. Recent developments in wireless technologies, and particularly low power wireless sensor networks, are just the type of advancements that may be adaptable to current and future safeguards monitoring needs.

Sensors have long been used to monitor nuclear materials throughout their life cycle. In the past these sensors have been hardwired or required a human operator. Wireless sensor networks would make item monitoring more practical, less costly, and reduce the need for on-site operation. Wireless sensors would be easier to setup, and more flexible in dynamically changing environments.

This paper discusses the necessary elements of a successful wireless platform for distributed radiation monitoring, and a proposed platform to meet these needs. The main focus of the work has been the power requirements, and a rough analysis of the proposed platform's battery life was completed. First, the paper will discuss some requirements for such a wireless platform, specific to the application of nuclear material monitoring. Then a detailed discussion of a proposed platform to meet these needs will be presented, and the analyses of the battery life along with the assumptions made in this calculation. Finally the paper will conclude with a discussion of some potential limitations of the design, and areas of future work.

Requirements

Each possible application of wireless sensor networks is unique and therefore has some unique design requirements that must be satisfied. In the area of nuclear safeguards there are some common elements to most applications. From these common elements, several requirements for a successful wireless sensor platform for use in distributed radiation monitoring activities have been identified. Many of these requirements create difficult tradeoffs that must be addressed early in the design process to make the platform feasible for use.

1. Power Considerations

Battery life and therefore power consumption is generally one of the biggest considerations in wireless sensor networks. Several methods of minimizing power consumption exist for wireless sensors, and most of these involve tradeoffs with various performance aspects. The tradeoffs generally involve wireless range and duty ratio. Duty ratio throughout the paper is used as the ratio of time in active operation to the idle time spent in very low power states. These same methods are applicable to nuclear safeguards.

For a wireless sensor platform to be practical for radiation monitoring, long battery life is essential. Many applications of wireless sensors would require the sensor nodes to be left unattended for 2-5 years or possibly longer. The usefulness of any sensor in this application will be limited by the length of time it can be left unattended.

2. Data Security and Authentication

Data security and authentication are necessary in nuclear safeguards applications to provide confidence in the monitoring activity. Specifically, authentication at the sensor level is desired to ensure the integrity of collected data. This is a requirement that is not always addressed in commercial wireless sensor network designs. Some form of effective encryption must be used to secure the data during transmission and to allow for authentication of the sender by the receiver. The problem is in the computational complexity of the common encryption methods. The encryption method must be implemented either in software or hardware. Software implementations are more flexible, but because of the computational complexity they usually require more processing power than low power embedded microprocessors can provide. This makes a hardware implementation preferred, but even a hardware encryption coprocessor will add to the power consumption of the sensor node.

3. Sensor Reports

Another important feature of the wireless sensor network is the method used to sample the sensor and report its data. Ideally, continuous sampling and reporting would be used to provide the real-time state of the environment [2]. This is not usually practical, because of the limited power of the sensor node. Alternative methods include periodic sampling, event driven, store and forward, and combinations of these methods [2]. Most radiation monitoring applications would require periodic sampling to track changes in background, and some event driven reports to collect data and relay data for certain alert conditions.

Proposed Platform

Some proposed solutions for the hardware, software, and network features of a wireless sensor network for distributed radiation monitoring are presented and explained. First, a choice of wireless standard along with the features of an appropriate transceiver are presented. Then the features necessary for the network topology and routing scheme are given. The necessary security features and a flexible method of reporting the sensor data are given. Finally an example of the sensor board and packaging are given from work done on a wired sensor platform.

One of the most important choices affecting the performance of the device in power consumption, flexibility, and cost is the choice of a wireless standard and transceiver package. Many wireless standards exist today along with a variety of transceivers with different characteristics. For nuclear safeguards, cost and availability become important issues. For these reasons standards that have a wide range of off-the-shelf components are preferred. The IEEE 802.15.4 standard for wireless personal data networks was selected because of its low power options and wide availability of off-the-shelf components. The IEEE 802.15.4 standard specifies the physical layer and medium access control on which the popular Zigbee communication protocols are based, and commonly operates at 2.4GHz both in the US and internationally. A TI CC2420 transceiver was selected based on its low power, low-cost, compliance with IEEE 802.15.4 and with the Zigbee standards, and its available AES coprocessor for use in encryption and authentication. The TI CC2420 is a single chip transceiver solution using

an SPI, Serial Peripheral Interface, connection to allow two way wireless communications from a microprocessor.

The topology used in a wireless sensor network can have a significant impact on the power consumption and fault tolerance of the sensor nodes. The topology with the lowest overall power consumption is the star type [2]. This topology uses significantly lower power than a mesh network based on its ability to operate at lower transceiver duty ratios. It doesn't however have the same fault tolerance or extended range of a mesh network [2]. Fault tolerance is probably an important feature in many radiation-monitoring scenarios to provide confidence in the security and dependability of the measurements.

The design proposed is capable of either network topology based on the software. A hybrid form of the two is suggested for testing using the platform. The hybrid form would allow the sensor nodes to setup ad-hoc star networks based on available routers. This would have only minimal impact on battery life, but would require additional routers to be used in networks needing better fault tolerance.

The limited range of the wireless signals would require routers, either wired or using a higher powered wireless standard, to be placed within signal range of the sensor nodes. A single router would be capable of handling an entire sensor network within its signal range. The smaller number of routers necessary would significantly reduce the cost of installing them as wired, and since they would not be sensor nodes they would also have more flexibility in placement. For example wired routers might be installed in convenient places in material storage areas, transport vehicles, and processing areas.

Sensors could then be attached to material containers, and could track radiation levels even as the container is moved about from location to location.

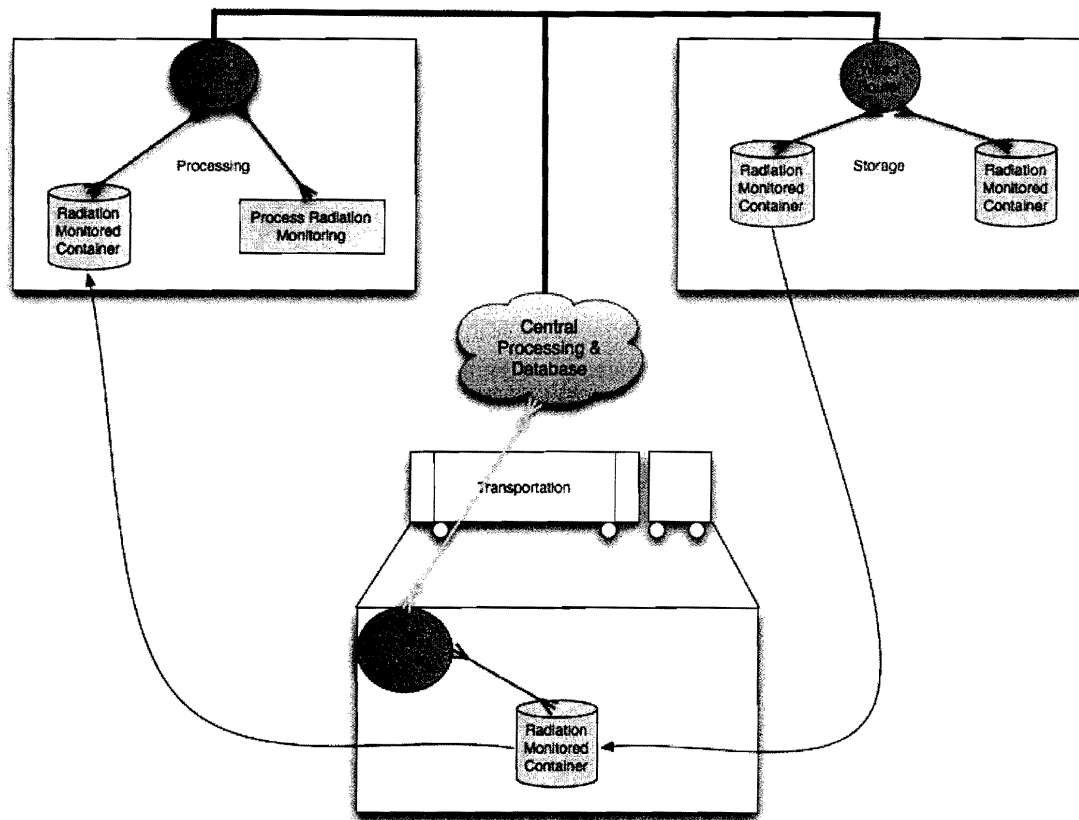


Figure 1: Network Architecture

The encryption and authentication of the system would need to be very robust for most monitoring situations. The proposed platform would use AES encryption as provided by the transceiver coprocessor mentioned above. The details of key management and authentication have not been worked out, and are topics for future work.

The sensor platform will allow for flexibility in choosing the sampling rate and duration, which will be set via software. Also, the inclusion of an accelerometer is proposed to allow the sensor to wake up from sleep to do additional monitoring during movement events. The rate with which the sensor reports the collected data would also be

software configurable allowing the tradeoff between more frequent reports and better battery life to be determined for individual applications.

This wireless sensor platform uses an interface for sensor that was defined on a previous Ethernet sensor platform [5]. This will allow sensor packages to be developed that will be compatible with both wired and wireless platforms. The Geiger Mueller sensor platform developed on the previous project has already been revised to allow for battery operation. This flexible and consistent interface will allow for a single sensor circuit design to be used with either platform as needed.

The packaging for the entire sensor package would be very much application specific. Shown below is a wired Geiger Mueller sensor package. The sensor board developed previously for this wired sensor platform could be reused with the wireless platform, and similar packaging could also be used. One of the advantages of the Geiger Mueller sensor is tolerance to higher temperature and virtually no drift in changing temperature environments. Some applications of this device might require packaging that would tolerate higher temperatures and more intense radiation than the plastic can withstand. Repackaging for these environments would be required.

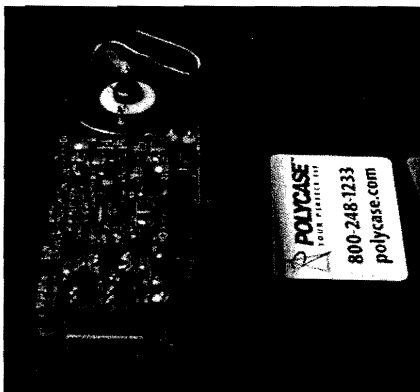


Figure 2: Previous Wired Geiger Mueller Sensor Packages

Battery Life Estimation

The platform proposed above was analyzed for probable battery life based upon the power consumption of each of the major devices. In order to reach this estimate some assumptions were made about the probable duty cycle of each of the components during normal use. Also the power consumption of the sensor circuit from the previous wired design was tested. Two different sampling durations for the sensor were considered.

In estimating the battery life of the device the main components will be the Geiger Mueller Sensor and its driver, the microprocessor, wireless transceiver, and accelerometer. The current ratings for each component were taken from their associated data sheet. The sleep current of each device was not considered in the calculation, because the sleep current of each of the devices is very low in the area of $10\mu\text{A}$.

The daily on time for each device was based on assumptions of normal use. The sensor was considered to sample 4 times a day with sampling durations of 5min. The accelerometer was assumed to remain active continuously in order to wake up the microprocessor in the event of motion. The transceiver would only become active after the sensor has collected data. It is assumed that 10 seconds would be more than sufficient to transmit the collected data under normal conditions. The microprocessor would remain active while the data was collected and transmitted.

Table 1: Estimation of Sensor Current Consumption

Device	Current Consumption(mA)	Daily on Time (h)	Daily Current Consumption (mAh)
Sensor 500V supply, GM Tube, and Output filter	12.6	0.333	4.1958
Accelerometer ADXL330	0.32	24	7.68
Microprocessor CY8C29466	5	0.349	1.745
Transmitter CC2420	18.8	0.01667	0.313396
Total Daily Current Consumption (mAh)			13.934196

Lithium Ion batteries were considered for the power source of the device. The supply voltage of the circuit was chosen to be 7.2V. This is 2 Lithium Ion batteries in series at their nominal voltage of 3.6V. The choice of 7.2V over 3.6V was related to the efficiency gain in the High Voltage DC to DC converter with a higher starting voltage. Both configurations of 2AA and 2C batteries were considered, and the associated battery life estimate is given in table 2.

Table 2: Battery Life Estimation

Battery	Ah(Nominal)	Battery Life(years)	
		5min. Sampling Duration	1min. Sampling Duration
2AA	2	0.39	0.59
4AA	4	0.79	1.18
2C	7.5	1.47	2.22
2D	17	3.34	5.04

Limitations

A wireless sensor can be vulnerable to attacks on its battery life. This is frequently referred to as a denial of sleep attack. The proposed platform would be vulnerable in several ways since it depends on low power sleep modes for its long battery life. One method used in these attacks is to provide intermittent or burst jamming to corrupt occasional bits during transmission [3]. This forces the sensor node to retransmit the entire packet; since the transceiver is the greatest power consumer of the device this can significantly reduce the battery life of the device. Another potential battery life attack for a wireless device would center on the accelerometer. If the device was kept in constant motion it could also significantly reduce the life of the battery. Both types of attacks could be detected and reported as abnormal events adversely affecting battery life, but more work needs to be done to identify effective methods for mitigating these attacks in relation to wireless monitoring.

The limited range of the sensor nodes and low transceiver duty cycle would limit the usefulness of the platform as a tracking device. The sensor node must have access to the wireless routers in order to communicate; outside of the router infrastructure the device can only record a limited amount of data internally. The device only transmits data to the router occasionally possibly as little as once or twice a day to conserve power. This means that the system won't recognize a sensor as missing until it fails to report. This could be up to 12-24 hours.

Future Work

Many of the specifications for distributed radiation monitoring via a wireless sensor platform have been proposed, but much work remains. The platform is in the final stage of design for radiation monitoring with a Geiger Mueller Tube. This platform can be tested for battery life and communication range. Additional testing will also include the necessary duration of sampling to achieve an accurate measurement of radiation levels. An analysis of the cost of the platform and its associated network hardware will also need to be completed.

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Introduction:

Nuclear receptors are ligand associated intracellular transcription factors involved in either up or down regulation of genes. They can be either located in the cytosol or nucleus of cells and over 65 have been discovered since 2002. They are important for intracellular signaling and gene regulation by activating or repressing gene transcription and participating in cellular “cross-talk.” They can serve as endocrine, paracrine, or autocrine signaling pathways depending on the ligand that activates them. Nuclear receptors are important for embryogenesis, homeostasis, reproduction, cell growth, and apoptosis as well as other physiological responses. Nuclear receptors can be characterized as either apo (without ligand) or holo (bound by ligand).

All nuclear receptors have similar structural organization. They contain an N terminal domain that harbors cell specific functions. This region is also important for post translational modifications like phosphorylation. Linked to the N terminus is a DNA binding domain (DBD) which binds DNA via various motifs like zinc fingers at different hormone response elements at the promoter region of the particular gene of interest. Zinc fingers are important for binding DNA as zinc ions are able to chelate two or four different cysteine residues thus allowing for protein-DNA interaction. DBDs also are sites for very weak dimerization interfaces only when bound to DNA. A hinge region connects the DBD with the ligand binding domain (LBD) and allows for flexibility of the protein and adoption of multiple conformations without any steric hindrance. The LBD is the site where ligands like hormones and steroids are able to bind at the ligand binding

pocket. Upon binding, conformational changes occur thus either recruiting activating or repression complexes which affect transcription appropriately. The LBD also contains the major interface necessary for dimerization with other nuclear receptors (heterodimers) or with itself (homodimers). However, some nuclear receptors can function as monomers. Dimerization allows for increased affinity, specificity, stability, and diversity as multiple sites are available for binding ligand and DNA. Finally, a C terminal domain is connected to the LBD but its function is unclear since it shows little evolutionary conservation like the other regions.

Retinoid X receptor is a nuclear receptor of utmost significance in physiological signal transduction. It can form homodimers with itself and has been described as a “promiscuous” nuclear receptor in that it forms heterodimers with many classes of nuclear receptors like, retinoic acid receptor (RAR), thyroid hormone receptor (TR), vitamin D receptor (VDR), constitutive androstane receptor (CAR), orphan receptors (those nuclear receptors with no known ligands), as well as others. The ligand for RXR is 9-cis retinoic acid which is a derivative of all-trans retinoic acid and promotes homodimer formation. These homodimers typically bind DR1 DNA response elements though their role physiologically is unknown. Some scientists believe 9-cis RA has too low of a concentration to be the endogenous ligand and think that it may be less potent ligand phytenic acid Three specific subtypes of RXR have been identified and isolated: alpha, beta, and gamma; heterodimer partners can for the most part bind any of the three and show no preference. RXR heterodimers are achieved by interactions with both the LBD and DBD. The DBD interactions dictate what type of DNA response element that the complex will bind while the LBD further strengthens the interaction. Upon binding

DNA, RXR is able to interact with several coactivators like SRC-1, TIF2, and ACTR which is ligand dependent through hydrophobic interactions. Little data suggest RXR interaction with corepressor so apo-RXR probably is a poor repressor of transcription. Given its ability to interact with a wide variety of nuclear receptors in several tissues, it can activate significant numbers of genes. RXR is an important nuclear receptor in determining certain diseases and treatments. Synthetic RXR ligands have been used to treat psoriasis, acne, cancer, obesity, and diabetes. With diabetes, the ligands decreased insulin resistance in mice with type II diabetes. Also studies in mutant mice have shown that in defective or absent beta RXRs there is poor dopamine signaling in the brain which could be linked to Parkinson's disease and schizophrenia. Mice with mutations in RXR gamma show resistance to thyroid hormone even without mutation with its heterodimer partner TR beta. RXR alpha is important for cardiac function and liver organogenesis while the beta form is significant in spermatogenesis.

Thyroid Hormone Receptor (TR) exist as four different isoforms alpha 1, alpha 2, beta 1, and beta 2. The alpha isoforms are encoded from chromosome 17 while the beta gene is located on chromosome 3. Depending on the tissue, either isoform may be expressed with some tissues allowing both alpha and beta receptors. TR alpha 1 and the beta receptors bind ligand with the highest affinity. They are active as homodimers, heterodimers, and monomers, and its ligand is triiodothyronine (T3) or thyroxine (T4, a less active form converted into T3 in circulation by three types of 5' monodeiodinases). T3 typically has a much stronger affinity for TR usually ten to fifteen orders of magnitude. T3 is important during development and defects cause growth disturbances and severe mental retardation (cretinism). In the apo form, TR can form heterodimers

with RXR and recruit corepressor. Part of the corepressor complex has histone deacetylase activity which further compacts chromatin turning expression off. Main corepressors included in the cellular machinery are NCoR, SMRT, and SUN-CoR. In the holo form when dimerized with RXR and in the presence of ligand, it is able to recruit a wide variety of coactivators. Typically this heterodimer binds DR4 response elements usually at a half site AGGTCA. TR is also able to bind other response elements composed of palindromes and everted repeats. Certain mutations mainly in the LBD of TR beta have been implicated in thyroid hormone resistance. Patients present with high levels of thyroid stimulating hormones so in turn also high levels of T3 and thyroxine. Therefore, the thyroid hormones are probably unable to negatively feedback on the anterior pituitary and suppress TSH release. The alpha one receptor is important for cardiac function and knockouts in mice show mice with low heart rates and prolonged QRS and QT peaks in EKGs. These mice also exhibit lower body temperature. If both alpha isoforms are absent, mice become increasingly hypothyroid with time and show stunted growth. The beta isoforms are particularly important for development of the auditory system. Knockouts of these genes cause phenotypically deaf mice because it is believed that this receptor is essential for ear maturation. Also complete inactivation of beta isoforms leads to hyperthyroxinemia.

Materials and Methods:

Throughout my time working in Dr. Fernandez's lab with Kumar, I have been focused primarily on the nuclear receptors thyroid hormone receptor and retinoid X receptor. The paradigm for each of these proteins comes from humans for RXR and chickens for TR particularly the alpha one isoform. We have experimented with several

vectors and cell lines for checking expression of the proteins and have discovered that pETSUMO in RIPL cells expresses best for RXR and pET15b in RIPL cells is most effective for TR expression. To purify the proteins, we have also experimented with a variety of conditions. After making glycerol stocks of the proper cells, we plate them on kanomycin/chloramphenicol plates for RXR since the cells confer chloramphenicol resistance while the plasmid carries kanomycin resistance. With TR, pET15b carries ampicillin resistance so they are plated on ampicillin/chloramphenicol plates as well. Optimal growth temperature is 37 degrees Celsius overnight. Following growth, a starter culture is made by innoculating a single colony using the same antibiotics for each in LB media. The starter culture is allowed to shake at 220 rpm at 37 degrees Celsius. Following growth in the starter culture, we inoculate 1% starter culture into 500mL aliquots of 2XYZT media and add antibiotics. The flasks are then allowed to shake at 37 degrees Celsius at 220 rpm until the O.D. reaches approximately 0.8. This absorbance is the optimal time for induction because the cells have entered an exponential phase which should yield maximum protein expression. After ideal optical density is achieved, the cells are cooled using an ice bath then induced with 0.5mM isopropyl-beta-D-thiogalactoside (IPTG). IPTG is a common inducer capable of binding lac repressor altering its conformation which decreases repressor affinity for operator. This decreased affinity allows for constitutive gene expression and increased mRNA production of our proteins. IPTG works at the level of the N subdomain. It alters this domain which in turn changes hinge association with the minor groove. Experiments have confirmed this theory; researchers engineered disulfide linkages between hinge helices which disrupted IPTG allosteric affects. Following induction, the cells are allowed to grow at 20 degrees

Celsius shaking at 220 rpm for approximately twenty hours. This time allows maximal protein expression within the cells. After this time period, cells are spun down at 6000rpm and can either be used for subsequent purification or frozen at -80 degrees Celsius and used later. In the past, we prepared samples and froze them for future use. However, we discovered that frozen protein particularly the TR cultures had significantly higher viscosities making them slimy and difficult to resuspend and lyse. Therefore, we started to use the cells fresh on the same day as purification which has yielded much better results. The cells are resuspended in a lysis buffer containing 50mM Tris pH 8 which keeps the pH fairly constant around the desired 8, 300mM NaCl which allows for a suitable cellular environment, 10% glycerol which also prevents non-specific binding. A complete tablet is added and resuspended which is a protease inhibitor which prevents any inherent proteases from degrading RXR and TR; the tablet also contains EDTA which acts as a chelating agent which quenches any unwanted metal ions present. 5mM Beta-mercapto ethanol is added to the lysis buffer just prior to resuspension and acts as strong reducing agent thus breaking any non-specific binding through cysteine residues and prevents reformation of any tertiary or quaternary structures. That exact concentration of BME is added to each buffer throughout the purification. 10mM DNase is also added to denature any DNA present that the protein might bind to. The cellular pellet is then resuspended in the aforementioned lysis buffer (usually about 25mL of buffer per 500mL of cells). We found that probably the most effective way to resuspend is with the use of the shaker. We previously manually resuspended by swirling and using spatulas and pipets. Using the shaker appears to be more time effective and efficient. Following resuspension, cells are lysed. We have tried various lysing methods; we had

used the French Press which lysed cells using high pressures previously but discovered a variety of problems. It appeared to generate excessive heat which could damage the protein. Also, if frozen cells were used, they exhibited a high viscosity and often could not be effectively lysed. Also, with the use of the French Press, some more concentrated volumes of cells should be used, and we started to do large scale preps consisting of 4.5L so French Press also was less time efficient. We opted to begin lysing cells using a sonic dismembrator. With sonication, we could use much more dilute solutions that were more conducive to our goals and another advantage is that sonication breaks any foreign DNA that DNase might not have denatured. The sonicator works by using sound energy to interrupt cell membranes thus releasing the intracellular components including our protein. We used a program designed for two minutes thirty seconds of pulse time; the program would pulse thirty seconds at seven power (which seemed to be ideal for lysis without generating excessive heat) and sit dormant for forty seconds. Following separate sonications of TR and RXR, cells were spun at 14k rpm at 4 degrees Celsius for forty-five minutes to allow the heavier cellular contents to collect at the bottom as a pellet thus leaving our proteins suspended within the supernatant. The supernatant was collected and pellet discarded. The pH was then determined of the supernatant and adjusted accordingly to achieve the desired 8. To pH, we used 3M Tris instead of NaOH which could denature our protein and HCL-glycine instead of concentrated HCL which can also lead to denaturation. We try to not use glycine unless the pH was well above 8 because precipitates form upon its addition. Its pH is also 2.2 which is significantly lower than our solution. If we add drops of glycine, it is initially localized before being disseminated throughout, and the region initially exposed to the acid experiences a drastic pH change

which could denature a portion of the protein. We then took the prepared TR and RXR supernatants to the cold room and loaded them onto Ni-NTA columns. Because of the excessive volumes often used for TR (often 4L) we used two columns. The columns were prepared by resuspending Ni-NTA beads in ethanol and adding two mL of the slurry (1mL bed volume) to the column membrane. The column was then washed with nano water to wash off the ethanol and then lysis buffer to equilibrate it. Only one column was used for RXR since we often only use 500mL because it expresses better. A 1.5 mL bed volume is used for it. The supernatants of both TR and RXR were allowed to run through the columns two times in the cold room. The colder temperatures prevent any possible denaturing; while running the supernatant twice ensures that almost all of the protein binds the beads though the majority should all bind beads with the first run. The Ni-NTA columns are a type of affinity chromatography where the supernatant with our protein binds the nickel beads through a histidine tag under atmospheric pressure. Ideally, anything that is not our protein should not bind and run through as waste. After the initial runthroughs and binding of protein to the beads, we washed each column with fifteen mL of lysis buffer and wash buffer (50mM Tris, 500mM NaCl, and 5mM BME) which removed any unwanted entities from the column as well as equilibrating it. We then wash the columns with 1X thrombin cleavage buffer usually about 10 mL which basically equilibrates the column thus readying the protein to be eluted. The elution buffer is composed of the wash buffer and 0.3 M imidazole. Because our proteins contain histidine tags, the high concentration of imidazole will knock the protein off the nickel beads and bind in its place. The reason for this is that histidine contains an imidazole group. The protein is generally eluted out in one mL increments and generally 6mL are

collected from each of the TR columns while 7mL is collected of RXR. Typically to elute, we add one mL of elution buffer at a time, swirl it, and allow it to sit for approximately 30 seconds. This ensures that imidazole will be exposed to more protein thus knocking it off and producing maximum yield. Once the proteins have been collected separately, they are spun down in eppendorf tubes at maximum speed as sometimes aggregates form following elution which we want to eliminate before dialysis. After the supernatant has been collected, a Bradford protein assay is done to determine protein concentrations. This assay is obtained by using 200 microliters of biorad solution, 800 microliters of ddH₂O, and 1 or 2 microliters of sample. By mixing the aforementioned components, we can obtain the optical density/absorbance of the protein. The spectrophotometer uses visible light at 595nm to determine O.D. Following calculations and comparison with a standard, we generally mix RXR and TR in equimolar concentrations though we have used different ratios such as 1.2 TR:1 RXR. Generally, on average, we obtain about 6mg of RXR per 500 mL of cells and around 3.5 mg of TR for 3L of cells. After mixing, which stabilizes the protein, in the desired concentrations, we load the protein into a dialysis bag. The dialysis bag has been prepared about an hour prior to its use. We cut it with scissors cleaned in ethanol and then place in nano water allowing the water to rid the bag of any impurities. The water is changed several times to ensure the bag is completely pure; this part is essential because the bag comes in direct contact with the newly formed protein and if any foreign substances remain on the bag like proteases the integrity of the protein is compromised. Following cleaning the bag with water, it is placed in a specially prepared dialysis buffer. The dialysis buffer (1L) is composed of 1X TCB like the elution buffer as well as DTT

which is a strong reducing agent. After the bag has become acclimated with its buffer, the protein can be loaded into the bag where we generally leave it for about an hour. Dialysis works by utilizing a semipermeable membrane and differing concentrations within the bag and in the buffer. Following elution, we typically have much higher concentrations of imidazole and salt which are bad for protein so dialysis is used as a means of diluting the protein. The salt and imidazole diffuse out of the bag into the buffer which is at a lower concentration than within the bag; soon equilibrium is reached. Our protein remains in the bag due to the selectivity of the dialysis bag. Our protein is larger than the pore size of the bag so it cannot diffuse. Following dialysis, we have the optimal conditions for our protein. However, the histidine tag and thrombin site still remain attached to the protein. To rid the newly formed heterodimer of these unwanted components, we do an hour of thrombin digestion though we have done it overnight before as well; an hour is sufficient to completely rid the protein of these tags. Normally we add about 120 microliters of thrombin. There is a thrombin cleavage site attached to the LBD. Attached to this thrombin cleavage site is the histidine tag. By bathing our protein in thrombin, the thrombin site as well as the histidine tag are cleaved off. Now we have a mixture of our desired protein but it also contains the cleaved tags. Prior to ridding the solution of these tags, we typically dilute the protein fourfold in a dilution buffer (100mL) that contains 2mM Tris pH 8.2. Now, we have a mixture composed of about 120 mL. We now eliminate the his tag-thrombin residues by running another single Ni-NTA column of generally about 750mL bed volume. The his tag will bind the Ni beads while our protein should flow through. The purpose of the Tris pH 8.2 is to make our protein more negatively charged. Typically, the TR/RXR heterodimer has an isoelectric point (pH

where a zwitterion exists) of about 7.5. The pI was determined from the primary structure of the protein so it is a slight misrepresentation since it is in its tertiary/quaternary structure so some charge is shielded but assuming 7.5 is relatively accurate. Assuming a pI of 7.5, any pH above that should make our protein more negatively charged thus allowing for anion exchange; this is why we use Tris at a pH of 8.2 though 8.2 is somewhat arbitrary as theoretically any pH above 7.5 should make our protein increasingly negative. After collecting the flowthrough from the nickel column, in theory we should have a solution purified with just our protein. To get better resolution and a more concentrated sample, we use anion exchange chromatography. The basic principle behind this technique involves loading our sample into an anion exchange column which is packed with negatively charged beads which our sample should bind. Any impurities in our protein that are at neutral pH or acidic pH will not bind. The protein is loaded using a buffer composed of sodium azide, a potent bacterostatic agent, and Tris pH 8.2. The protein is then eluted out of the anion exchange column using a similar buffer of Tris 8.2 and NaCl. The high salt concentration knocks the protein off of the beads which can then be collected. Typically after anion exchange, we have a concentrated sample of protein usually about 2mL. We are able to detect when our protein is eluted by the use of ultraviolet radiation which the protein absorbs. Finally, the protein is subjected to one final purification step known as fast protein liquid chromatography (FPLC). This separates protein based on size. A column is packed with beads that have pores in them. If the protein is small, it will move very slowly down the column because it becomes stuck in the pores and is eluted much later than larger proteins which have no problem traversing the column without getting stuck. The particular column that we use for the

full length TR/RXR column is an S-200 meaning that proteins up to 200 kilodaltons can be effectively separated. The heterodimer has an approximate weight of kDa with TR being about kDa and RXR about kDa. The buffer used to elute from this particular column is composed of 20mM Tris pH 8, 200mM NaCl, 5mM magnesium chloride, and 1mM TCEP (or some other reducing agent like DTT especially when the protein will be used for crystallization).

Recently we have noticed that TR has seemed to be bound by or associated with another high molecular weight protein, possibly a heat shock protein, which has caused less expression. Physiologically, if this interference is in fact a HSP, TR would never be bound to one since it is always within the nucleus of eukaryotic cells. For our experiments, however, we use E. coli cells which are prokaryotes so association with a HSP is feasible. We have tried to add ATP in the past which seemed to produce little effect thus essentially eliminating the prospect of this molecular band protein from being a HSP since ATP should cause it to break from our protein. Therefore, we tried a different approach when preparing our initial starter cultures. We initially prepared the same media and starter cultures. We ran an expression check under six different conditions. Growth prior to induction was preformed as we had; however, to half of the flasks, we added 20 micromolar T3 to the media. All conditions were allowed to grow under the same conditions (37 degrees C and 220rpm) until O.D. reached approximately 0.8. After optimum growth conditions had been met, each flask underwent different conditions before and after induction. The conditions are as follows:

Growth Temperatures (Celsius) Before Induction	Expression Temperatures (Celsius)	Other Conditions
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37	37 for 5 hours	normal
37	20	sit at room temp. before induction
37 (with T3)	37 for 5 hours with T3	T3 to lysis buffer, 1X TCB, elution
37 (with T3)	20 with T3	T3 to lysis buffer, 1X TCB, elution
37	20	Transfer to shaker before induction, lysis buffer with Triton X100
37 (with T3)	20 with T3	slow cooling, T3 to lysis buffer, 1X TCB, elution, TritonX100

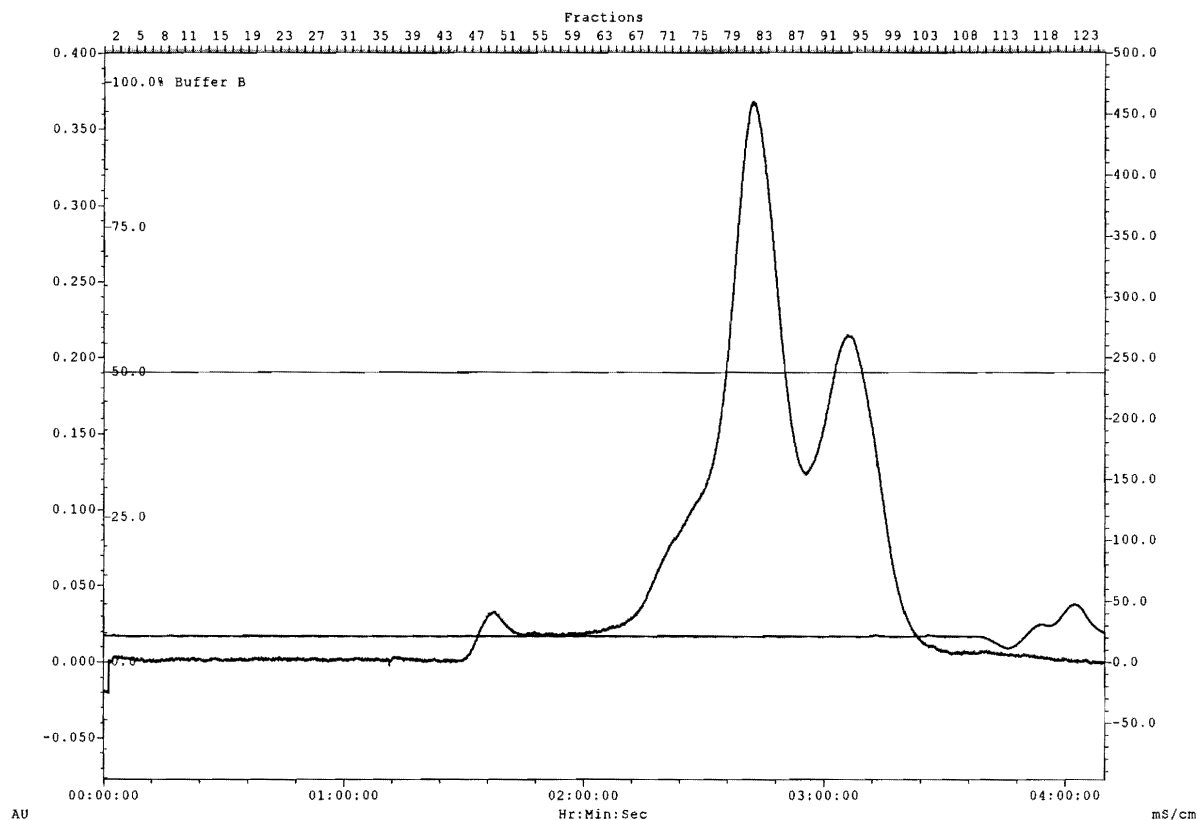
We were checking a variety of things with this expression check. Some of which include the addition of T3 at each subsequent step of the protein prep, the temperature following induction, how the flasks were cooled prior to induction, and the addition of triton X100. After induction, two flasks grew at 37 for only five hours. The reason for this is that the higher temperature facilitates cellular growth, and if grown the normal twenty hours that we do, the cells would surely enter the death phase as they would grow exponentially exhausting all the nutrients making them unviable. Triton X100 was added to see if the addition of a powerful detergent would have any effect on expression. The detergent should interrupt any hydrophobic interactions that TR formed with other proteins.

After all conditions had been prepared, we ran an expression check. We did this essentially like a normal protein prep as I described in the previous paragraphs. We were careful keeping each of the six samples separate to prevent any cross-contamination. Differences from the normal protein prep include the use of only 500 mL bed volume for Ni-NTA columns and we eluted out 2 mL total from each column (12 total) in 250 microliter increments. We then estimated protein concentrations using a Bradford protein assay to determine protein concentrations. We then ran a 12% SDS PAGE gel to get definitive results from the expression. After running the gel, it was determined that the when T3 was added to the media and each step as well as growing at 20 degrees Celsius after induction produced significantly better expression results in terms of purity and expression.

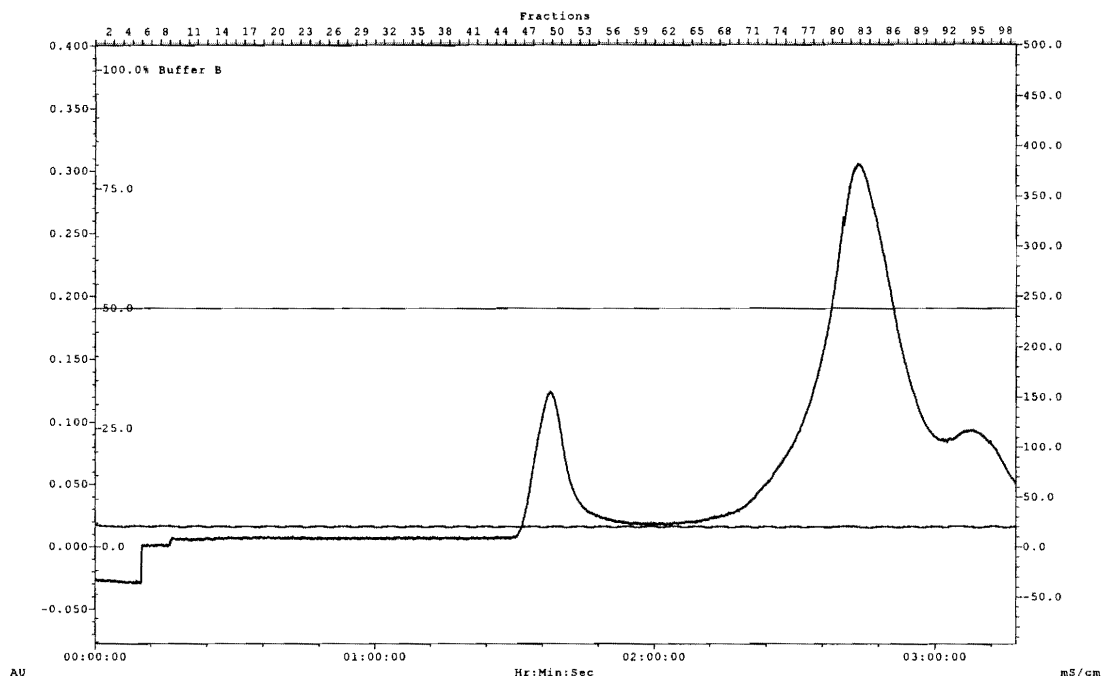
After determining optimal preparation protocols, we performed another heterodimer prep using only two liters of TR with our new technique. The prep was performed under the exact same conditions as before which I described in the first paragraphs. We found that the purity of the protein was better as well as the yield. We obtained about 3.5 mg of TR with 2L following our new protocol as compared to 3.5 mg per 3L under our old conditions.

We have also recently created a construct where we transformed TR and RXR into a bisistronic vector pET15B. The quality is much better than our full length protein but cloning both full lengths proteins into the same vector does not give good results since the proteins are much larger.

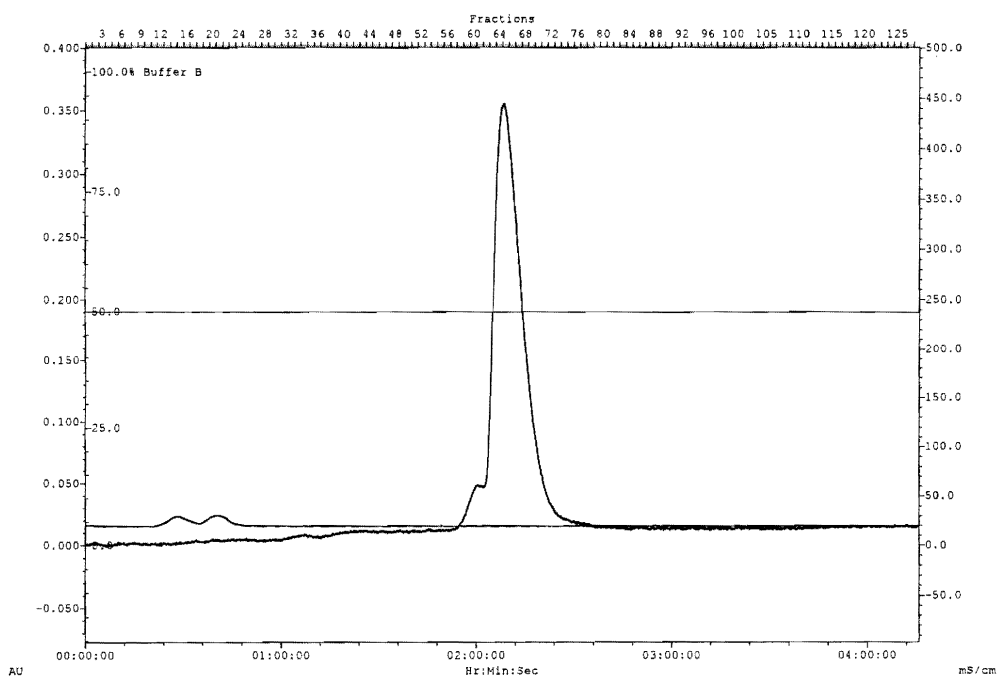
Results:



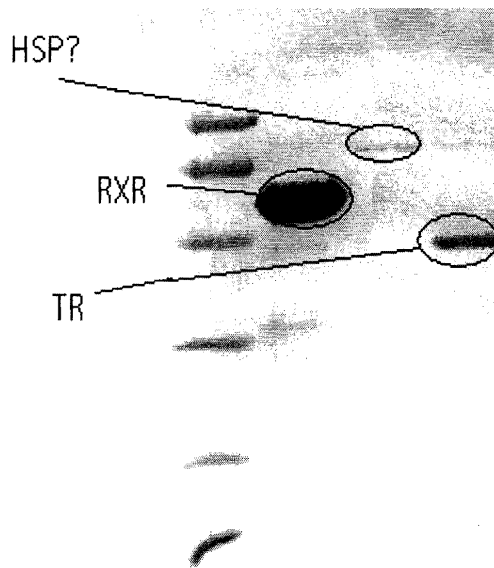
The above chromatogram was taken following a prep on October 16, 2007. The largest peak between fractions 79-83 is our heterodimer. The protein can be collected and then undergo further experimentation like isothermal calorimetry, fluorescence, crystallization, etc. In some instances depending on our goal we add different DNA sequences, ligand, etc. to the protein. The peak that follows the heterodimer that begins around fraction 90 is probably a monomer of either TR or RXR since it is smaller and eluted later that we want to eliminate; this can be done by trying to make sure that we mix the proteins in exact concentrations so that all will heterodimerize leaving only our desired protein. The slight peak occurring around fraction 45 is a high molecular weight protein that is possibly sequestering some of our protein or it could be something like an RXR tetramer. We try to eliminate this peak by using ATP and T3 in later experiments.



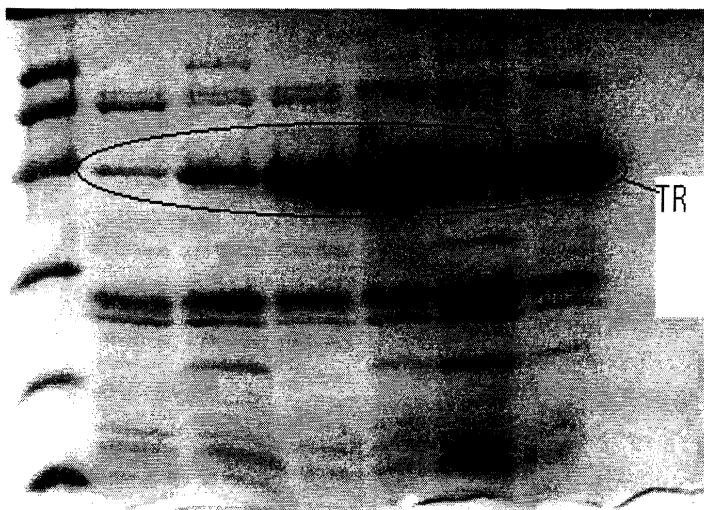
The above chromatogram was taken on January 16, 2008. We still get good heterodimerization and seem to nearly eliminate the later peak which gives more quality protein, but the initial peak is much larger suggesting more interaction with either a HSP or other protein not cleared during the purification.



This chromatogram shows TR/RXR LBDs. Following concentration, the LBDs show particularly good separation, quality, and yield. This heterodimer protein is good for crystallization but not DNA binding since it has no DBD.



This 12% SDS PAGE gel was taken from different points during a protein purification. The first lane is a ladder that shows known samples that can be used for comparison. The second lane is a sample of RXR taken following elution from Ni-NTA column. The third lane is the high molecular weight protein that is possibly a heat shock protein. Finally the last lane contains the same band as in lane three as well as a TR sample taken after gel filtration. This particular gel shows that the potential heat shock protein associates with TR rather than RXR which is why we started adding ligand. To definitively determine the structure of this unknown protein we would have to do mass spec and compare with known values which would probably allow us to determine a way which would not allow it to associate with our protein.



Growth Temperatures (Celsius) Before Induction	Expression Temperatures (Celsius)	Other Conditions	SDS PAGE gel lane
37	37 for 5 hours	normal	2
37	20	sit at room temp. before induction	3
37 (with T3)	37 for 5 hours with T3	T3 to lysis buffer, 1X TCB, elution	4
37 (with T3)	20 with T3	T3 to lysis buffer, 1X TCB, elution	5
37	20	Transfer to shaker before induction, lysis buffer with Triton X100	6
37 (with T3)	20 with T3	slow cooling, T3 to lysis buffer, 1X TCB, elution, TritonX100	7

The previous 12% SDS PAGE gel shows the results of our TR alpha 1 expression check under various conditions. Lane one corresponds to a ladder while the other lanes are represented by the conditions listed in the table above. It is apparent from the gel that lane five expresses TR best by the thickness of the band. The other bands are irrelevant and mostly probably degradation products.

Discussion:

It is clear that nuclear receptors are vital in many physiological responses; mutations in as few as one amino acid can lead to dire phenotypes. Particular nuclear receptors often used as paradigms are TR and RXR which are both significant in most tissues. Finding optimal conditions to purify these proteins is imperative for further experimentation and thus learning how they interact and function. We have several

different protocols that we utilize to purify proteins but no one has proved perfect yet. We are in the process of designing a new protocol with TR untagged to bind with a his tagged SUMO RXR. Because the apparent high molecular weight band protein appears to only associate with TR, by not using a tag, any TR that does not dimerize with RXR should flow through leaving no chance for association with the possible heat shock protein. This should allow RXR to be fully saturated with TR and only the heterodimer should form.

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