

Single particle imaging of molecular trafficking in neurons

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In this project, we aim to elucidate neuronal cell physiology at the molecular level by visualization of molecular trafficking processes involved in neuronal communication. Neurons communicate by secreting peptides, hormones, and neurotransmitters, which involves exocytosis, and receptor mediated endocytosis. We will use single-molecule tracking by using nanoparticles, which is uniquely suited for studies of molecular trafficking implicated in exo-and endocytosis. We will tag enzymes released in exocytosis with highly visible and photostable nanoparticles, which will be followed over long periods of time all the way through to subsequent endocytosis involving the enzyme. Similarly, labeled receptor ligands will render visible the neurons with the targeted receptor over extended periods of time. Consequently, the fate of both dendritic or axonal vesicles, and their physiologically significant pathways, e.g. transportation to the nerve terminals, will be followed. We propose to apply the nanoparticle methodology to study molecular trafficking of a neuronal enzyme DBH in the cell in pursuit of a medically relevant objective of neuron-neuron communication in relation to blood pressure regulation. By visualizing/tracking DBH we will be able to determine how cellular catecholamine handling is altered in C1 neurons in hypertension.

Successful demonstration of endocytosis-mediated internalisation of nanoparticles will provide a solid platform to explore a possibility of cargo delivery into the cell, where the nanoparticles serve as cargo vehicles. Such new way of delivery of drugs, gene, or peptides will open a new way to control cell functions, and, specifically it will open the possibility to develop new treatment methods by improved control of neurons involved in the regulation of arterial pressure.

On this backdrop, we will carry out research into production, characterisation, and single-particle imaging application of novel nanoparticles. These include luminescent nanodiamonds and gold nanoparticles, which will be fabricated within this project, and we will also use innovative commercial quantum dots. Biochemical protocols of the nanoparticle surface modification, and bioconjugation with antibodies, will allow targeted labelling of specific molecular species on the neuronal membrane. Expertise in biochemistry is essential for the success of the proposed collaborative team, and will be sought by requesting funding for a suitably qualified postdoctoral research associate and by recruitment of HDR students with a suitable background.

Unraveling of neuronal response to various physiological stimuli at the molecular level will provide new insights and potentially uncover new functionalities of this extremely complex cellular system. Novel nanoparticles and single particle imaging strategies will be instrumental in uncovering communication and regulation functionality of neurons. We expect that our project will lay a foundation for future broader studies of neuron-neuron communication by integrating efforts in the areas of neuronal physiology nanotechnology, biochemistry, and biophotonics.

The project presents a unique opportunity to:

- Make a major advance in the areas of nanotechnology, single-particle imaging, and neuroscience, emerging frontier research areas highly emphasised in leading scientific journals,
- Target a medically relevant problem with a view to producing information readily translated to future medical therapies, and
- Leverage the availability of solid expertise in the area of photonics, nanotechnology, and neurosciences, existing at the Macquarie University and help establish new synergies.

8. Project description (four page maximum)

Background, aims, significance and innovation

The central nervous system plays a critical role in the management of blood flow to the tissues and its return to the heart and lungs. The complex array of cell groups within the brain and their intercommunication has been a central focus of research of Goodchild and colleagues who advanced the understanding of intricate control of sympathetic pathways by various neurotransmitter systems. One major unresolved question is whether these neurotransmitters are released by the cell. To answer this we will focus on two mechanisms of neuronal communication, neuronal exo- endocytosis. These will be studied in brain regions essential in the control of blood pressure where cell targeted drug delivery could be implemented.

Single-molecule tracking is uniquely suited to studies of molecular trafficking implicated in exo-and endocytosis. Tagging an exocytosis-released enzyme with a highly visible and photostable nanoparticle will permit visualization of the endocytosis event involving the enzyme. Such fluorescent nanoparticles render the enzyme tagged vesicle or ligand visible in the cell for extended periods of time. Consequently the fate of both dendritic and axonal vesicles and their physiologically significant pathways, e.g. transportation to the nerve terminals, can be followed. The challenge is to be able to engineer the nanoparticle so that it can be successfully internalized. This involves optimizing the nanoparticle size, surface charge and ways of attaching other molecules. It is important to note that successful demonstration of the nanoparticle internalisation, as e.g. shown in Figure, will open a way for targeted drug delivery into the neurons via nanoparticles attached to a cargo of therapeutic agent(s).

Catecholamine cells are one of the specialised cell groups in the brain, which are involved in a diverse range of critical functions. One such cell group, called the C1, is critical to the tonic and reflex control of arterial pressure. This project will focus on the understanding the functioning of C1 neurons, and the fate of dopamine beta hydroxylase (DBH), important for neural function. Tagging this enzyme with a photostable optical label will make it possible to follow the recycling of DBH, its further recruitment for the next exocytotic process, or transportation along the neuron axons to the nerve terminals. Visualising this process in living neurons will enable us to explore important questions such as: under what conditions is noradrenaline released from these neurons, or does dendritic release of noradrenaline or adrenaline occur? Answers to questions such as these may ultimately make it possible to establish the molecular basis of blood pressure control.

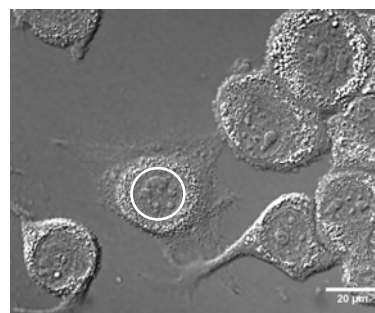


Figure. Optical image of scattering nanodiamonds transfected through the cells visible as bright rims around the dim nuclei. White circle outlines the nucleus. (As published by AZ and co-authors).

Aims:

1. We will engineer novel nanoparticles and develop strategies suitable for imaging single molecules responsible for communication and regulation functionality of neurons, e.g. dopamine beta hydroxylase. We will examine the following:
 - a. Luminescence nanodiamonds (LUMINAD) that are small (4 nm in size), non-cytotoxic, and particularly easy to bioconjugate.
 - b. Quantum dots (QDs), well suited for background-free optical imaging and single molecule tracking.
 - c. Gold nanoparticles with plasmonic resonances producing excellent visibility in cells.
2. We will develop chemical protocols for the nanoparticle surface modification, so that targeting biomolecules are bound to nanoparticles surfaces and specifically bind targeted molecular species on neuron membranes.
3. Once developed, the nanoparticles will be deployed for single particle tracking to elucidate the neuronal activity at the molecular level.
4. Optically bright, surface-functionalised nanoparticles will be also explored as potential drug delivery carriers.

Why this is significant and cutting edge science:

Visualization of molecular trafficking processes will increase our, so far incomplete, understanding of neuronal cell physiology at the molecular level. Direct imaging of synthesis, storage, recruitment, and recycling of physiologically active biomolecules will contribute to improved understanding of neuronal response to various physiological stimuli at the molecular level. This will provide new insights, and

potentially uncover new functionalities of this extremely complex cellular system.

The project will bring significant advances in understanding how the C1 cell population operates in setting the tonic and reflex control of blood pressure. Determining how specific neurochemicals including catecholamines are handled by individual neurons and identifying precisely how this is altered in disease conditions such as hypertension will be a substantial contribution to the field.

We expect that our project will initiate a broader study of neuron-neuron communication by integrating efforts in the areas of neuronal physiology nanotechnology, biochemistry, and biophotonics available at Macquarie University. We expect further integration of these advances into the medical research.

We propose to apply the nanoparticle methodology to study molecular trafficking of a neuronal enzyme DBH in the cell in pursuit of a medically relevant objective of neuron-neuron communication, and following from this study, exploring potential of nanoparticle-assisted neuronal cellular drug delivery. To the best of our knowledge, such a study has not been undertaken elsewhere.

Research plan, methods and timetable.

Development of novel nanoparticles providing high optical contrast with respect to cellular background represents the key challenge of the project. Nanotechnology methods which allow engineering of nanoparticles and making them suitable for single biomolecule tracking, including design of the important properties such as size, shape, surface charge, and controllable surface functional groups. These are now discussed in detail.

Luminescent nanodiamond represents a novel nanoparticle almost ideally suited for intracellular imaging and, potentially, drug delivery. Its wide application has been triggered by the recent advances in production of truly nanometre-sized nanocrystals and creation of nitrogen-vacancy defects that render them fluorescent, and, therefore, detectable on the often autofluorescent cellular background. We will produce LUMINADS using the detonation/disintegration method yielding 4-nm nanocrystals, carried out within current collaboration with NanoCarbon Research Institute, Japan (Prof Osawa). As a backup strategy, we will rely on creation of colour centres in commercial ND products containing larger size nanocrystals of 50–100 nm. This will be realized by irradiating ND samples with a high-energy electron beam resulting in creation of vacancies in NDs. Production of luminescent ND will be accomplished by annealing in vacuum at a temperature of 600–1000°C allowing migration of crystal vacancies, embedded in NDs during the production, to the vicinity of the substitutional nitrogen.

Bioconjugation of ND will take advantage of oxygen-containing functional groups on the surface of high-temperature oxidised NDs. *AZ et al.* have previously developed a chemical protocol based on water-soluble derivatives of 1-Ethyl-3-(3-dimethylaminoethyl)carbodiimide hydrochloride (EDS) and N-Hydroxy-sulfo succinimide (sulfo-NHS), which relies on the presence of carboxyl groups on the ND surface. The new chemical reactivity of the surface made it possible to attach several types of biomolecules, such as bovine serum albumin (BSA) and ATP. The same procedure will be repeated in this project.

The surface-functionalised LUMINAD will require conjugation with an antibody against DBH. When conjugated, the suspension will initially be applied to brain and spinal cord thick slices (400 µm) and maintained in a chamber perfused with oxygenated tissue culture medium. These slices are commonly used in electrophysiological investigation of cellular function. It is also possible to preload the cells by injecting the conjugated nanoparticles directly into the brain and activating the neurons physiologically to determine internalization prior to isolating the brain slice. C1 cells will be visualized independently by prior labeling with fluorescent microbeads microinjected into the spinal cord to permit rapid targeting of the tissue site of interest. Similarly in spinal cord sections sympathetic preganglionic neurons, which contain the terminals of C1 neurons will be preloaded by injection of nanoparticles in the adrenal gland. As a result of exocytosis of vesicles (from nerve terminals and potentially cell bodies), membrane bound DBH will be exposed to the extracellular environment. The antibody will bind to the DBH and when endocytosed the nanodiamond will end up in the cell. Trafficking of the tagged DBH will be visualized under multiphoton or conventional confocal microscopy. The tagged cells can be activated or inhibited using a variety of drugs including glutamate/gaba/peptides to determine how the tagged DBH behaves under different physiological conditions. This cell entry process is feasible as anti-DBH conjugated toxins (e.g. saporin) kill DBH containing cells selectively, whereas unconjugated toxin cannot move into the cell. This process of cell entry has been described for both the cell body and the terminals of catecholamine neurons. Using a brain slice preparation will also permit populations of cells to be visualized to determine if different cells within the same cell group handle the tagged nanoparticles differently. Our findings in individual or populations of neurons can be compared in brain slices from normotensive commonly available Sprague Dawley rats and from the

spontaneously hypertensive rat (SHR).

In order to utilise the full potential of novel LUMINAD optical labels for molecular trafficking studies, we will make use of real-time time-gated optical imaging techniques available at MNRF (University of Sydney). In this technique, gating is synchronised with an optical excitation from an ultrashort pulsed laser, e.g. of picosecond pulse width, the fluorescent nanoparticle lifetime can be accurately measured by processing the detected fluorescence signal acquired versus the delay between the excitation and detection circuits. More importantly, if the lifetime of a nanoparticle of interest is much greater (or shorter) than that of the unwanted fluorescent background, the proposed system provides very efficient discrimination from this background, enabling high-contrast imaging of the selected fluorophore distribution in the sample. LUMINAD represents another important fluorophore with long luminescence lifetime, which makes it possible to achieve background-free optical imaging on the single-particle level.

Gold nanoparticles will be fabricated by using the femtosecond laser ablation method. Recently, it has been discovered that ablating target material placed in a solute by using a femtosecond pulsed laser enables production of nanomaterial (nanoparticles) with unique and controllable properties. These include size, surface charge, and surface functional groups. The latter property is particularly important for development of biochemistry procedure of attachment of DBH antibody that will be used to tag a DBH enzyme.

Quantum dots are bright and photostable fluorescent nanoparticles that featured prominently in biology research in recent years. We will utilise maturity of this technology, and, specifically, well developed biochemical procedures for QD surface modification with carboxyl, amine and polyethylene-glycol functional groups. We plan to procure commercially available biochemically-specific QDs to develop and deploy the DBH conjugation protocol. Single-molecule imaging, including application of the time-gated detection scheme will be tested and optimized using these nanoparticles.

Outcomes of the research, including how the project positions the group for future research in the area.

The project will explore a significant neurobiological question of whether or not there is dendritic release of neurotransmitters. Furthermore, these studies will also produce new markers for cells that are activated by specific neurotransmitters. Identification of molecular pathways during neuron-neuron communication specifically initially those involved in C1 neurons and identification how catecholamine handling in individual neurons differs in hypertension will be a major outcome.

These will provide a strong foundation for further studies of mechanisms and molecular pathways within the neural system during neuron-neuron communication. The study can be extended to include angiotensin receptors which belong to large array of G-protein coupled receptors (GPCR) that are found in the central nervous system. The advantage of targeting these receptors is that they are internalized together with their bound ligand upon activation thus permitting access to the inside of the cell. We will explore how angiotensin receptor tracking differs in hypertension and by seeing single cells we will be able to determine if more receptors are recruited to single cells.

This improved understanding will lead to the possibility of better biochemical control of neuronal communication. In this context, the anticipated demonstration of nanoparticle-assisted drug delivery into neuronal cells will directly pave the way to future therapeutical approaches.

The project outcomes in the area of nanotechnology and bioimaging will include the development of novel nanomaterials and methods suitable for imaging molecular trafficking in neurons. We will also have demonstrated novel background-free optical imaging methods adapted to a specific biomedical imaging context.

Research contribution to the national benefit

Hypertension is a major predictor for cardiovascular disease and stroke. Hypertension is now found in over 20% of the population, and although several therapies are available more than 70% of identified hypertension is poorly controlled. This project will provide novel insights into the molecular basis for hypertension via application of specialised nanoparticles, which, at the same time, have the potential as drug delivery carriers.

The proposed research will make a strong contribution to the Australian science, where it will improve our standing in neuroscience and biophotonics. It will also have impact on research areas, which use single-molecule imaging. It will enable key advances in biology of nerve cells, with implications in medicine, including neuronal cell communication research aiming at treatment of

relevant disorders. Because of these implications, the project falls into the National Research Priority National Research Priority Promoting and Maintaining Good Health, Research Goal, Preventive Healthcare.