Quantification of ovarian cancer markers with integrated microfluidic concentration gradient and imaging nanohole surface plasmon resonance

Carlos Escobedo,a Yu-Wei Chou,b Mohammad Rahman,b Xiaobo Duan,c Reuven Gordon,d David Sinton,e Alexandre G. Brolo b and Jacqueline Ferreira∗f

Nanohole array-based biosensors integrated with a microfluidic concentration gradient generator were used for imaging detection and quantification of ovarian cancer markers. Calibration curves based on controlled concentrations of the analyte were created using a microfluidic stepped diffusive mixing scheme. Quantification of samples with unknown concentration of analyte was achieved by image-intensity comparison with the calibration curves. The biosensors were first used to detect the immobilization of ovarian cancer marker antibodies, and subsequently applied for the quantification of the ovarian cancer marker r-PAX8 (with a limit of detection of about 5 nM and a dynamic range from 0.25 to 9.0 µg.mL−1). The proposed biosensor demonstrated the ability of self-generating calibration curves on-chip in an integrated microfluidic platform, representing a further step towards the development of comprehensive lab-on-chip biomedical diagnostics based on nanohole array technology.

1 Introduction

Biomarker detection and quantification is an important tool for early cancer diagnosis.1 In addition, quantitative monitoring of biomolecular interactions is appealing for many biomedical applications and usually critical for those involving gene expression, virology, cell signaling and pathogen detection.2−5 Specifically, biomarker detection involves different types of interactions, such as specific and non-specific protein–protein binding as well as protein interactions with other molecules such as metabolites, nucleic acids and drug molecules.6−8 These interactions and the dynamic characteristics of the binding events are studied using quantitative biosensors.9−10

Received 5th November 2012
Accepted 9th January 2013
DOI: 10.1039/c3an36616b
www.rsc.org/analyst

Cite this: Analyst, 2013, 138, 1450

1 The Royal Society of Chemistry 2013

In the past few decades, intensive efforts have been made towards the development of microfluidic-based biomedical diagnosis platforms that integrate several functions on-chip. The benefits offered by microfluidic-based technologies are well-documented11 including their potential integration into portable point-of-care (POC) and point-of-use (POU) diagnostic systems.12 Biomarker quantification in microfluidic-based systems requires the use of standard methods and calibration for each test. Calibration curves are extensively used in traditional analytical chemistry13−14 and have been incorporated into microfluidic platforms in the past15−17 as they have concentration gradient generators.18−20 Thus, the concept of a microfluidic system with the capability of both generating calibration curves and carrying out the analysis simultaneously on the same chip is particularly attractive. The implementation of this concept requires detection elements that are suitable to the microfluidic environment and that allow both good sensitivity and multiplexing. Nanohole arrays in metal films fulfill all these requirements and can be employed in combination with microfluidic systems. Im et al. (2012) recently demonstrated improved spectral resolution and simultaneous quantification of antibody-ligand binding kinetics using an affordable device comprising parallel microchannels over a single nanohole array.21 We employ nanohole arrays integrated in microfluidic channels as the biosensing strategy in this work.

Nanohole arrays in metal films support surface plasmon resonances (SPR)22−24 that enable extraordinary optical transmission at resonant wavelengths.25,26 EOT occurs at wavelengths that allow the excitation of surface plasmons (SPs) from the periodic nanohole arrays by grating coupling. As a result,
nanohole arrays mediate the enhanced transmission of light at those wavelengths. Surface plasmons (SPs) are oscillations of charges at the metallic interface that are sensitive to changes in the refractive index environment close to the metal surface. In other words, the adsorption of species at the metallic surface will alter the conditions for SPR excitation, leading to changes in EOT. The measurement of these changes in the light transmitted by the nanohole structures can be achieved using simple optical arrangements involving low cost light sources and detectors. This characteristic of nanohole arrays is relevant for designing biosensors that can be easily integrated into miniaturized devices.\textsuperscript{27–29} Analogous to the commercial SPR sensors, nanohole array-based sensing is most applicable to the label-free detection of biological species. This major advantage has motivated the use of nanohole arrays (and other types of SPR systems) in biomedical applications.\textsuperscript{30–32} Nanohole array SPR based biosensors can also be implemented in imaging mode.\textsuperscript{33,34} This detection scheme is based on the measurement of changes in the intensity of monochromatic light transmitted through the nanohole arrays due to molecular adsorption. The main advantage of this detection scheme is the possibility of multiple analyte detection in a single acquired image (multiplexing), in addition to the label-free real-time quantitative detection, which is common to all types of SPR sensors. Integration of nanohole arrays into microfluidic chips has been reported in several studies.\textsuperscript{10,35–41} More recently, the development of biosensors based on nanohole arrays has focused on the implementation of flow-through architectures that combine plasmonic sensing with advantages of transporting the analyte through nanochannels and on new analyte concentration schemes.\textsuperscript{42–46}

All the advantages afforded by a nanohole-based biosensor integrated in microfluidic systems suggest that these devices can be explored for the early detection of diseases. The early detection of cancer, for instance, is critical to effective treatment and survival. For example, a 95% survival rate has been reported for the early detection of ovarian cancer, whereas late detection is almost always fatal.\textsuperscript{47} Hence, the need for serological screening is particularly acute for ovarian cancer which is generally asymptomatic until late stages, being one of the hardest to diagnose at early stages.

The detection of CA-125 in blood samples by the enzyme-linked immunosorbent assay (ELISA) is a serological test used to help diagnosis and to follow treatment progression of ovarian cancer.\textsuperscript{48} Recently, several new ovarian cancer biomarkers have been identified, potentially providing more alternatives to complement the CA-125 tests and improve the reliability of serological diagnosis of ovarian cancer. For instance, the transcription factor PAX8 is highly expressed in epithelial ovarian cancer but absent in the same cells of healthy individuals.\textsuperscript{49} Based on these developments, we demonstrate in this work the quantitative label-free detection of the ovarian cancer biomarker PAX8 using an array of nanohole arrays integrated into a microfluidic concentration gradient generator. The approach is to generate, in every assay, a calibration curve on-chip for the quantification of a sample of unknown concentration, which is measured in parallel. The approach of detecting an analyte using a calibration curve obtained in the same platform is regularly used, in a macroscopic scale, in common ELISA tests (both the sample and the calibration are measured in the same well plate). In our particular case, the concentration gradient for the calibration curve is achieved by stepped diffusive mixing of two base streams with known analyte concentrations. Each of the diluted streams generated by the mixing process is directed along six outlet microchannels flowing in parallel with a microchannel containing the sample to analyze. Four in-line nanohole arrays were used for sensing each concentration of biomarker at the outlet channels, as well as for the individual sample channel. The quantification of biomarker concentration in the sample stream is achieved by SPR imaging via normal transmission of monochromatic light on a CCD detector array. As the amount of bound analyte to the nanohole arrays is proportional to the analyte concentration in the specific outlet microchannel, the transmitted light through the nanohole arrays in different microchannels also varies. These intensity variations are tracked and the determinations of unknown analyte concentrations are achieved by comparison with the standard curve. The approach presented here is a further step towards comprehensive affordable POC and POU biochemical diagnostics.

2 Materials and methods

2.1 Nanohole array fabrication

Nanohole arrays were fabricated in commercial glass slides (EMF, Ithaca, NY) coated with 100 nm thick Au and 5 nm thick Cr adhesion layers by focused-ion beam (FIB) milling. For milling, the ion beam was set to 30 keV with a beam current of 30 pA. The typical beam spot was 10 nm, and the dwell time was 2 ms. A white light transmission spectrum for one of the arrays is shown in Fig. 1.

A total of 28 nanohole arrays were fabricated in an arrangement consisting of 7 columns and 4 rows with a total footprint of less than 1.0 mm by 0.4 mm. The fabricated arrays were inspected by scanning electron microscopy (SEM). Fig. 2

![Fig. 1 White light transmission spectrum from one of the nanohole arrays milled through the gold film. Hole diameter and array periodicity are 200 nm and 420 nm, respectively. The peaks in the transmission spectrum are due to EOT. The dashed line corresponds to 632.8 nm, which is the wavelength of the laser used in the imaging experiments.](image)
shows the SEM images from nanohole arrays detailing the nanostructure.

2.2 Fabrication of microfluidic concentration gradient generators

A replica molding technique was employed for the fabrication of the microfluidic concentration gradient generator. This technique is widely used in microfluidic applications and it is described in detail elsewhere.28 The general steps during the fabrication procedure are described next. A mask with the microfluidic pattern was generated using CAD software. The design included three inlets and two outlets of 1.5 mm and seven 90 μm wide microchannels, with a total footprint of 22 by 22 mm², as shown in Fig. 3. Next, a master was fabricated by spin-coating SU-8 50 photoresist (MicroChem Corp., Newton, MA) onto a clean 3 inch silicon wafer (Silicon Quest International Inc., Santa Clara, CA). The coated wafer was then pre-baked at 65 °C and 95 °C for 1 and 6 minutes, respectively. The mask with the microfluidic pattern was then placed over the coated wafer and exposed to UV light for 90 seconds. Next, the exposed wafer was hard baked at 65 °C for 1 minute and at 95 °C for 10 minutes. The master was subsequently developed using a SU-8 developer (MicroChem Corp., Newton, MA). A 14 : 1 mixture of Sylgard 184 elastomer to curing agent (Dow Corning, Midland, MI) was mixed, degassed in a vacuum and poured onto the master. After baking at 85 °C for 20 minutes, the replica was removed from the mould. Inlets and outlets were provided 1 mm punched holes for fluidic access. Polyetheretherketone (PEEK) tubing (Upchurch Scientific, Oak Harbor, WA) was used for fluidic connections. Microscrew syringe pumps (Harvard Apparatus, MA) were used to infuse the solutions into the microchip. The walls of the microfluidic chip were coated with 0.1 mM BSA solution to avoid non-specific adsorption. The characterization of the concentration generation chip was achieved by fluorescence microscopy using a Leica DMi6000B epi-fluorescent microscope (Leica, Germany) and an Orca AG CCD camera (Hamamatsu, Japan). Images were acquired at a rate of 2 frames per second and an exposure time of 400 milliseconds.

2.3 Experimental setup for SPR imaging

The microfluidic chip was placed on top of the nanohole arrays substrate, with the columns of nanohole arrays aligned with the outlet microchannels, as shown schematically in Fig. 3. An acrylic top plate was used to mechanically seal the system. A Helium–Neon (He–Ne) laser (MellesGriot, Carlsbad, USA) with emission at 632.8 nm was used as the excitation source and directed on the nanohole arrays at normal incidence. As shown by the dashed line in Fig. 1, 632.8 nm is close to one of the EOT maxima for these arrays. Two 20 × working distance microscope objectives (Leica Microsystems, Wetzlar, Germany) were used to expand the beam uniformly over the nanohole arrays. The transmitted light through the arrays was acquired using a cooled CCD camera (PhotometricsCoolSNAP HQ2, Austin, USA). The CCD images were acquired using a software-controlled shutter and an integration time of 20 milliseconds. The transmitted light intensity was measured by using the ImageJ software. One row of nanohole arrays was selected at a time with rectangles. The area of the selection rectangle was the same for all the rows. The integrated intensities of the nanohole arrays inside each rectangle were obtained by the ImageJ software. The intensity values were arbitrary and normalized using the intensities of the light transmitted through the nanoholes exposed to only phosphate buffer saline (PBS) solution.

2.4 Surface modification and detection

A simplified scheme for the nanohole modification and protein detection is presented in Fig. 4. The nanohole arrays were cleaned in an ultrasonic bath for 3 min, rinsed with ethanol and with water, and dried with nitrogen gas before experiments (Fig. 4a). Next, to allow chemisorption of the biomolecules via amide linkages, a dithiobisuccinimidyl undecanoate (DSU) monolayer was assembled on the arrays by immersing the sample in 2 mM DSU in dimethyl sulfoxide (DMSO) for 72 hours (Fig. 4b). The construction of the capture antibody substrate was completed by flowing the antibody solution over the nanohole arrays (an SPR image of the surface was then acquired). Prior to the detection of the antigen, the surface was treated with a blocking agent such as bovine serum albumin (BSA) and ethylamine to cap the unreacted succinimidyl groups. The modified surface with the immobilized antibody is shown in Fig. 4c. The modified surface was then exposed to solutions containing the target protein [r-PAX8], which binds preferentially to the immobilized antibody, as shown in Fig. 4d. The protein adsorption altered the intensity of the laser light (632.8 nm) transmitted through the arrays.
2.5 Chemicals

DSU (92.7%) was purchased from Dojindo (Dojindo Molecular Technologies Inc., Japan). DMSO was purchased from Caledon (Caledon Labs, Georgetown, ON). Sodium chloride (99%), sodium phosphate dibasic (99%) and potassium chloride (99%) were purchased from ACP (ACP Chemicals Inc., Toronto, ON), and fluorescein was purchased from Invitrogen (Invitrogen Corp., Carlsbad, CA). Paired box gene 8 (PAX8) and the antigen r-PAX8 were provided by the Trev & Joyce Deeley Research Centre of the British Columbia Cancer Agency (Victoria, BC).

3 Results and discussion

3.1 Microfluidic gradient characterization through fluorescence

Fig. 3 shows a schematic of the microfluidic chip used in this work. The chip included a microfluidic network with 2 inlets branching and increasing the number of channels by one at each dilution step, ending in 6 independent microchannels with a common outlet. This splitting and mixing approach provides a passive and accurate method of generating mixed concentration fluid streams. PBS solution was infused in one of the inlets and 1 mM fluorescein, dissolved in PBS, into the second inlet. A flow rate of 5 µL.min⁻¹ was used for both inlets. The laminar regime characteristic of microfluidics allows co-laminar flows, where mixing is dominated by cross-stream diffusion. In this chip design, the lengths of the microchannels were calculated, based on the required residence times, to ensure full mixing of the encountering streams. At each mixing step and throughout the entire microfluidic network, the outer channels transported the original concentrations from the inlets and the mixture in the middle channels had a volume ratio of 1 : 1. Therefore the dilution factors for the six channel chip were calculated as: 1 : 0.9375 : 0.6875 : 0.3125 : 0.0625 : 0. These factors were used to estimate the analyte concentrations flowing in each channel over the surface of the nanohole arrays.
upon adsorption, which were useful properties in the preliminary experiments. The generation of a fluidic concentration gradient generator is demonstrated by the fluorescence image of the 6 outlet channels, as shown in Fig. 5.

A separate single channel with 1 inlet and 1 outlet ran parallel to the 6 outlet channels from the network and was used to transport the sample fluid containing the analyte of unknown concentration (see Fig. 3). Fluorescence microscopy was used to characterize the dilution scheme resulting from the microfluidic concentration gradient generator (Fig. 5). The images were 1324 by 1024 pixels and had an 8-bit dynamic range. Figure 5a shows a fluorescence image of the 6 outlet microchannels under chip operation. Figure 5b shows the normalized fluorescence intensity (20 by 20 pixels) versus the channel confirming the gradient of relative concentrations generated by the chip. The sigmoidal shape of Fig. 5b arises inherently from the splitting and combination of the preceding streams, as shown in Fig. 3.

3.2 Ovarian cancer antibody immobilization

After calibration through fluorescence imaging, the nanohole arrays were then aligned with the microchannels. As shown in Fig. 3, the channels were identified from 1 to 6 in addition to the sample channel, and the rows were identified from A to D. The SPR sensitivity of our system was first tested simply using the non-specific immobilization of the ovarian cancer antibodies. This is not the most adequate method of immobilization since it leads to a distribution of orientations, affecting the antibody binding capability due to steric effects with respect to the spatial orientation of the capture antibody. Nevertheless, this system allowed irreversible immobilization and large intensity changes upon adsorption, which were useful properties in the preliminary tests and optimization of the sensing platform. In this case, the microfluidic chip was first used to create a concentration gradient from a 27 μg.mL⁻¹ PAX8 antibody solution in PBS (pH 7.5). One of the inlets was provided with the PAX8 antibody solution (channel 6) while channel 1 contained PBS in the absence of the antibody. Both solutions were transported by the microfluidic chip at a fixed flow rate of 10 μL.min⁻¹. This resulted in 6 different concentrations at the outlet channels flowing over the 6 columns of nanohole arrays. At the same time, in the sample channel, a blind test was run by flowing a PAX8 antibody solution at an unknown concentration to the experimenter. The microfluidic configuration allowed parallel reading of the 7 microchannels at the detection zone (Fig. 3). The inset in Fig. 6a shows the SPR image acquired with the CCD camera after the introduction of the PAX8 antibody solution. The monochromatic light transmitted through each of the nanohole arrays appears as “red spots” in Fig. 6. The presence of adsorbed species at the nanohole surface shifts the conditions for SPR resonance, affecting the intensity of the transmitted light. The changes in intensity are then proportional to the amount of proteins adsorbed at the surface. The transmitted light intensity through the arrays varies with the concentration of the antibody. The intensity of 632.8 nm transmitted light through nanohole arrays with 420 nm of periodicity is expected to decrease with a corresponding increase in the refractive index of the surrounding medium, as observed in Fig. 6. It is important to point out that even the refractive index of the most concentrated antibody solution (27 μg.mL⁻¹) was very similar to the PBS solution (1.3333 at 22.1 °C); therefore, the transmitted light intensity differences observed in the inset of Fig. 6a cannot be assigned to differences in the bulk refractive index of the solutions, and the observed results originate from the changes in the refractive index at the gold–solution interface due to the immobilization of PAX8 antibody.

Fig. 6a shows the normalized intensities for 1 row of arrays (row B – see labels in Fig. 3) across all channels, including the sample channel. The maximum normalized transmission intensity occurs in microchannel 1 (ca. 10 pixels in the x-axis), where the arrays were in contact with PBS solution. Fig. 6b shows the average normalized intensity calculated using all the arrays in a given column. This comparison reveals a concentration of ca. 24.85 ± 0.05 μg.mL⁻¹, which is in close agreement (within about 5%) with the nominal concentration of the sample (23.70 μg.mL⁻¹). The effective refractive index associated with the protein immobilization was estimated by using a procedure described by Jung et al. (1998). The refractive index of the biomolecular layer was estimated to be 1.57. An equivalent calibration curve in terms of effective refractive indices is presented as an inset in Fig. 6b. Therefore, a decrease of ca. 80% in normalized transmitted intensity (relative to PBS solution) was observed for an increase of 0.0063 in refractive index units (RIU).

3.3 Ovarian cancer marker detection

The previous section demonstrated the surface sensitivity of our platform for non-specific surface binding of representative biomolecules. In this section, the efficiency of the platform in affinity assays will be explored. A DSU monolayer was assembled on the nanohole arrays, as described in the previous section, and the PAX8 antibodies were then immobilized...
homogeneously on all the nanohole arrays to provide the targets for the ovarian cancer marker protein r-PAX8. This was achieved by introducing the same concentrations (27 μg.mL⁻¹) of PAX-8 antibody on both inlets, followed by treating the surface with a blocking agent (as illustrated in Fig. 4). After the surface modification, the microfluidic chip was flushed with PBS to clear the excess PAX8 antibody and blocking agents. A concentration gradient of r-PAX8 antigen was then introduced by using an 8.50 μg.mL⁻¹ r-PAX8 solution in PBS in one inlet and only PBS in the other. The solutions were introduced into the channels at a constant flow rate of 10 μL.min⁻¹; and the images were acquired after 15 min of flow. Fig. 7 shows the SPR images from the PAX 8–r-PAX8 affinity assay. Blind tests were performed by flowing in the sample lane r-PAX8 solutions with concentrations unknown to the experimenter. Typical nominal concentrations of the r-PAX8 samples ranged from 2.00 μg.mL⁻¹ to 5.30 μg.mL⁻¹, to probe both ends of the concentration gradient limits. The insets in Fig. 7 show the SPR images obtained after the introduction of the r-PAX8 calibration solutions and the “unknown” r-PAX8 samples. The unknown concentrations were determined from the calibration curve presented in Fig. 7c. The calibration presented a reasonable linearity ($R^2$ around 0.93), although the overall performance of the sensor deteriorated relative to Fig. 6. This occurred mainly for two reasons: (i) the SPR sensor is more sensitive to near surface events. The r-PAX8 was still within the SP evanescent field when interacted to an immobilized protein layer of PAX8, but its position further away from the surface decreased the SPR response; (ii) the absolute amount of transmitted light was smaller for the experiments in Fig. 7, relative to Fig. 6. This means that the noise level in Fig. 7 was larger, which reflected in larger variations. In order to minimize these issues, the measurements from Fig. 7 were carried out three times by recovering the sample before each run with an acidic solution (pH 3.0).

The smaller variation in intensity for the images in Fig. 7 relative to that observed for the PAX8 antibody immobilization experiment (Fig. 6) can be further related to: (i) differences in coverage of the r-PAX8 and PAX8 antibody in both experiments; and (ii) the fact that the r-PAX8 molecule is smaller than the PAX8 antibody. Consequently, the immobilization of the PAX8 antibody on the biosensor has a larger impact on the effective refractive index at the gold–solution interface, resulting in higher intensity contrast due to the adsorption. As discussed above, the linearity of the calibration curve in Fig. 7c ($R^2$ = 0.93) was not ideal. This is probably due to a combination of factors, including the small molecular size, increased instrumental noise and the lack of stability of the instrumentation to small variations (including temperature). Moreover, the dynamic range of this method is only about one order of magnitude. In principle, the dynamic range was dictated by the linear gradient dilution chip. The dynamic range could be extended by using more complex dilution microfluidic chips that allow logarithmic concentration steps. It is noteworthy that the dynamic range will be limited at the lower end by the LOD and at the upper end by fast surface saturation.

Analyzing the normalized intensities of the arrays for two different sample concentrations (Fig. 7a and b), it is possible to notice that the maximum transmitted light intensity, as expected, was observed in channel 1 (first channel from left to right, as identified in Fig. 4), corresponding to the PBS solution and the minimum intensity was observed in channel 6, corresponding to the most concentrated solution of r-PAX8. The concentrations of the unknown samples were calculated by comparison to the concentration calibration curve (Fig. 7c), using the same procedure as in Fig. 6b. The experimentally determined concentrations for r-PAX8 were 1.54 ± 0.74 μg.mL⁻¹ and 6.47 ± 0.46 μg.mL⁻¹ for the two samples as shown in Fig. 7, respectively. The deviation of these concentration values relative to that expected from the unknown samples was about 23% in both samples. The unknown sample concentrations were validated by other analytical methods (ELISA and Biacore SPR). The results were within 5% of the nominal value for ELISA and about 20% of the nominal value for the Biacore SPR method. Another aspect of Fig. 7 is the reasonable standard deviation calculated from the results of the 4 arrays in the column (less
than 10% variation). These are a good indication of the reliability of the assay.

Concerning clinical biomarker detection, serum concentrations of cancer biomarkers are expected to be in the pM to nM-range.34 A typical level of r-PAX8 in patients with cancer is still being determined,35 but it is expected that the concentrations should fall within that typical range. The limit of detection (LOD) for r-PAX8 determined from our calibration curves is around 5 nM. This level of performance of the imaging nanohole surface plasmon resonance biosensor developed in this work is comparable to other biosensors for cancer detection.36–62 However, the sensitivity can be improved to reach the target range by either improving instrumentation or by moving to more specialized nanohole-based sensing schemes, such as biaxial in-hole array detection with flow-through sample delivery.40,42,63,64

4 Conclusions

In summary, a SPR-based biosensor integrated into a micro-fluidic concentration gradient generator was demonstrated for the quantitative assessment of ovarian cancer biomarkers. Sensing was achieved by SPR imaging of 28 nanohole arrays (arranged in 7 columns), simultaneously. The ovarian cancer biomarkers were quantified using a microfluidic concentration gradient integrated with the nanohole sensor platform. This allows the generation of an “in situ” calibration curve simultaneously with the analysis. The system was tested using “unknown” samples prepared in our laboratory by different experimenters. The limit of detection for r-PAX8 protein achieved in this work was about 5 nM within a dynamic range of about 1 order of magnitude. The dynamic range was limited by the linear range of the dilution chip used to generate the calibration curve.

The logical next step for this technology is its direct implementation using clinical samples. Towards that goal, the current system would benefit from improved instrumentation (implementing temperature control, low-noise detectors, and highly stable light sources). Importantly, the proof-of-concept operation demonstrated in this work emphasizes the potential of the nanohole technology for parallel, label-free and real-time quantification of biological interactions.

Acknowledgements

This work was supported by operating grants from NSERC (including discovery grants and a strategic research grant with the BC Cancer Agency Trev and Joyce Deely Antibody Research Unit and MicralyneInc) and from the NSERC Strategic Network for Bioplasmonic Systems (BiopSys). C.E. also thanks NSERC for a postgraduate fellowship and J.F. thanks the Canadian Bureau for International Education-Department of Foreign Affairs and International Trade (CBIE-DFAIT) of Canada for a Graduate Student Exchange Fellowship. Equipment grants were provided by the Canada Foundation for Innovation (CFI), the British Columbia Knowledge and Development Fund (BCKDF) and the University of Victoria through the New Opportunities and the Canada Research Chairs Programs.

References

2. I. Hellstrom, J. Raycra