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# Implementation of a Flow Cytometry Strategy to Isolate and Assess Heterogeneous Membrane Raft Domains

Morgan F. Khan, Tammy L. Unruh and Julie P. Deans  
*University of Calgary  
Canada*

## 1. Introduction

Flow cytometry is an analytical technique based on the detection and quantitation of scattered light from individual cells or particles. This is achieved by channelling particles single file past a light source and collecting the scattered light with detectors and filters positioned at specific angles. This has enabled the identification of unique cellular characteristics and is widely used in the field of medical science in both clinical and research labs to study biological phenomena including apoptosis, cell cycle progression, cell surface protein heterogeneity, and calcium signaling (Brown & Wittwer, 2000, Krishan, 1975, Vermes et al., 2000). Generally, flow cytometers are optimized to study cells with 1-30  $\mu\text{m}$  diameters, with investigations of smaller cells and particles only recently becoming more widely applied. This coincides with the commercial availability of flow instruments capable of detecting synthetic beads as small as 200 nm in diameter.

Flow cytometry analysis of small particles can be performed on standard instrumentation. However, particles that fall at or below the wavelength ( $\lambda$ ) of the incident light beam exhibit different scatter behaviour than the diffraction and interference typical of larger particles (Shapiro, 2003, Zwicker, 2010). As a result, an accurate, quantitative measure of particle size below the  $1\lambda$  threshold is not possible. In addition, any sample that contains heterogeneous particles with a large size spread will be susceptible to different scatter characteristics making comparisons between these particles impossible. Despite these limitations, flow cytometry may be used for characterisation of sub-micron particles when a definitive size measure is not essential, provided the particles under investigation are subject to similar scatter characteristics. Biological applications where sub-micron flow cytometry analysis has been utilized successfully include the characterization of circulating microparticles (Abrams et al., 1990, Jy et al., 2010), and the characterization of bacteria and viruses in aquatic systems (Lomas et al., 2011). Another area of research that could benefit greatly from flow cytometry analysis and sorting is the study of plasma membrane microdomains called membrane rafts. These microdomains are essential mediators of plasma membrane function, yet they remain difficult to characterize by conventional techniques.

## 2. Visualizing protein heterogeneity within membrane vesicles

Initially described by Simons and van Meer (Simons & van Meer, 1988), membrane rafts are regions within eukaryotic plasma membranes created by preferential packing of saturated long-chain fatty acids and cholesterol, and implicated in diverse cellular functions, including signaling and membrane protein trafficking (Gupta & DeFranco, 2007). The liquid-ordered environment of raft domains attracts a subset of proteins largely distinct from those in the more fluid surrounding membrane. Additionally, membrane rafts are now known to be heterogeneous with respect to protein content. Intracellular raft heterogeneity is a useful model to explain the regulation of the many different signaling pathways orchestrated by the plasma membrane; only the specific subset of proteins required for a given biological process is recruited to a common region. Although raft protein heterogeneity is presumably essential to effect specific biochemical outcomes, the proteomic differences among distinct membrane microdomains have been difficult to characterize as a result of inadequacies associated with currently available biochemical tools.

Raft-based protein heterogeneity can currently be visualized on live cells with microscopy-based techniques such as fluorescence microscopy, single particle tracking and image correlation spectroscopy (Dietrich et al., 2002, Gupta & DeFranco, 2003, Lenne et al., 2006, Mutch et al., 2007). Protein motion and relative proximity to other molecules are tracked and the extent of co-localization between them dictates the likelihood of molecules residing in common raft domains (Gupta & DeFranco, 2003, Petrie & Deans, 2002). Microscopy has been extremely valuable in identifying intracellular raft protein heterogeneity; however, overlap of emission spectra between individual fluorophores limits the number of labels that can be simultaneously observed. In addition, microscopy can only be performed on known proteins and identification of unknown protein components within a distinct raft population requires a biochemical strategy.

Proteomic raft analysis can be achieved by extracting these domains from the plasma membrane (Foster et al., 2003, Jordan & Rodgers, 2003, Lin et al., 2010, Mannova et al., 2006, Nebl et al., 2002). Isolation of membrane rafts was traditionally performed in cold, non-ionic detergent, such as Triton X-100, in which raft proteins are largely insoluble (Brown & Rose, 1992). However, detergent extraction of model membranes was shown to artificially induce raft coalescence, therefore the use of detergent extraction for the purpose of characterizing intracellular heterogeneity is now generally avoided (Munro, 2003, Shogomori & Brown, 2003, Wilson et al., 2004). An alternative to detergent-extraction involves cell lysis by mechanical disruption, followed by density centrifugation to isolate the rafts to the buoyant fraction (Macdonald & Pike, 2005, Smart et al., 1995, Song et al., 1996). Membrane rafts extracted in the absence of detergent are thought to better reflect raft composition *in vivo*, although these isolates suffer from substantially greater contamination from non-raft proteins (Foster et al., 2003). Regardless of the isolation procedure, heterogeneous populations of membrane rafts are contained within a common sample. Consequently, differences between unique populations of rafts can only be achieved by enriching specific raft subsets from the biochemical isolate. There are currently no available purification schemes suitable for the investigation of dynamic, sub-microscopic cellular components. Thus, adapting flow cytometry detection and sorting to the analysis of membrane rafts would be a major advance in characterizing intracellular protein heterogeneity.

### 3. Visualizing protein heterogeneity within membrane vesicles

The extraction of membrane rafts from B lymphocytes was performed as described (Polyak et al., 2008). Briefly, cells were lysed by mechanical disruption and the lysate was mixed with an equal volume of 50% Optiprep solution (dark grey region in Figure 1a), followed by an overlay of 20% then 5% Optiprep solutions (light grey and white regions in Figure 1a, respectively). After centrifugation to equilibrium, the resulting step gradient was sampled at the two gradient interfaces where cellular material was observed; the 25%-20% interface (heavy membranes), and the 20%-5% interface (light membranes), as shown in Figure 1a. The light and heavy membrane fractions, as well as an unfractionated sample, were imaged by electron microscopy to evaluate the nature of the enriched cellular material (see Figure 1b-c). The heavy membrane fraction contains more organelles (ribosomes and mitochondria) and few vesicles, whereas the light membrane fraction contains sealed vesicles of varied size in addition to other cellular debris.

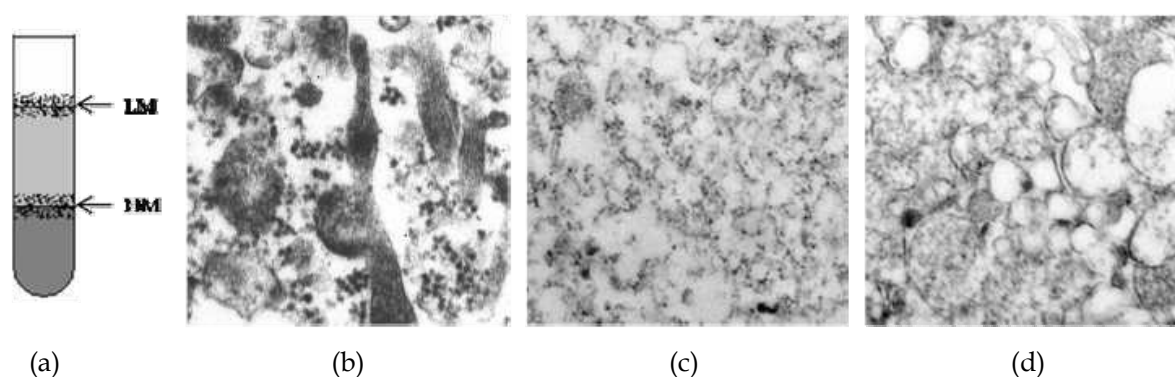


Fig. 1. Transmission Electron Microscopy of cellular material shows enrichment of vesicles in the buoyant fraction of density gradients. BJAB B cells were mechanically lysed and subjected to gradient centrifugation. The step gradient and the location within the gradient where cellular material was confined are shown in (a). The light membrane (LM) and heavy membrane (HM) fractions were collected at the interfaces of the gradient steps. Transmission electron microscopy was performed on (b) the unfractionated material, (c) the heavy and (d) the light membrane fractions

The light membrane fraction was also observed by fluorescence and light microscopy to confirm that these vesicles are derived from the plasma membrane. BJAB B cells were labelled with the lipophilic membrane stain Vibrant-Dil. Differential Interference Contrast (DIC) and Dil-stained images from whole cells and light membranes extracted from these cells are shown in Figure 2. Imaging of whole B cells shows the successful incorporation of Dil into the plasma membrane and minimal intracellular staining. The extensive overlap between the DIC and Dil images of the light membrane fraction indicates that the majority of the vesicles are of plasma membrane origin.

To assess whether distinct membrane raft domains can be observed within the light membrane vesicles, we selected two pairs of raft-associated proteins that have been shown previously to be largely dissociated on intact cells: CD20/ surface IgM (sIgM) and CD20/linker for activation of B-cells (LAB). The degree of co-localization of sIgM and CD20 previously reported on intact BJAB cells stimulated for 1 minute was 47% (Petrie & Deans, 2002); co-localization of CD20 and LAB on intact cells was 25% (Mutch et al., 2007). BJAB

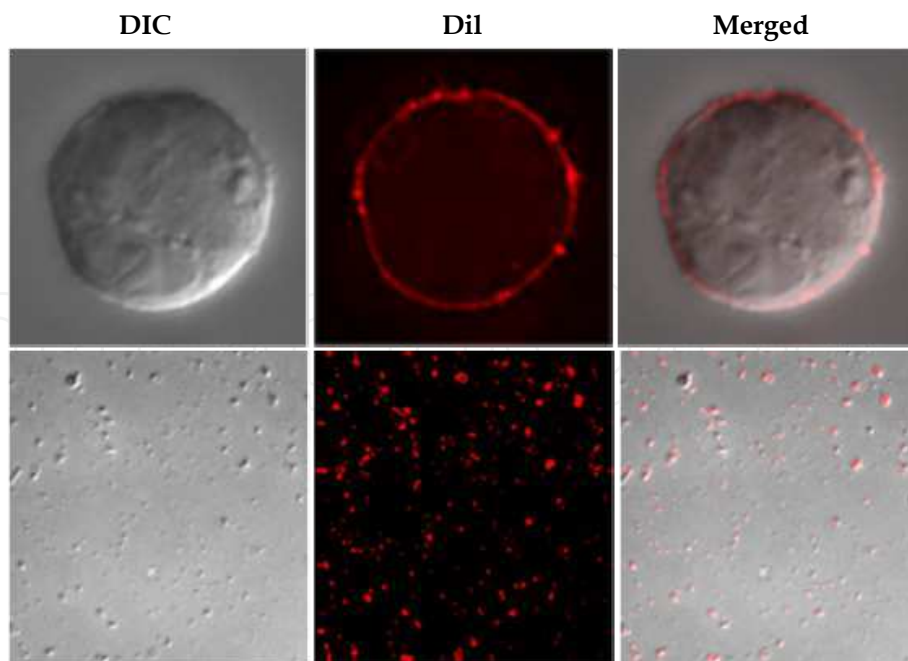


Fig. 2. Vesicles observed in the light membrane fraction originate from the plasma membrane. Whole B220 cells were labelled with Vibrant-Dil and either imaged directly (top panels) or processed to obtain the light membrane fraction (bottom panels). The DIC images (left) and fluorescence images (centre) were merged (right)

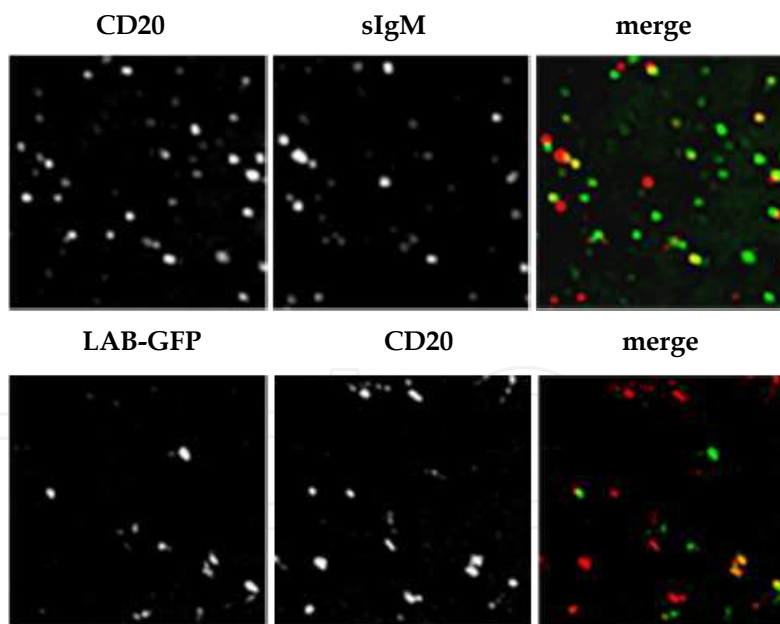


Fig. 3. Distinct membrane rafts are preserved in vesicles contained within the light membrane fraction. Membrane raft protein pairs, CD20/sIgM and LAB/CD20, were selected for analysis as these have limited co-localization in whole cells. For the CD20/sIgM analysis, B220 cells were labelled with anti-IgM-PE and anti-CD20-AF488. For the LAB/CD20 analysis, B220 cells that expressed LAB-GFP were labelled with anti-CD20-AF555 (bottom panel). Light membranes isolated from both sets of labelled cells were analyzed by immunofluorescence microscopy. The percentage co-localization was 49.4% for CD20/sIgM, and 30% for LAB/CD20, in agreement with values previously obtained from whole cell analysis



cells were labelled with anti-IgM-phycoerythrin (PE) and anti-CD20-Alexa Fluor 488 (AF488) for the first protein pair. The second pair utilized BJAB cells that expressed LAB tagged with green fluorescent protein (GFP) and labelled with anti-CD20-AF555. Once labelled, the light membranes were extracted and imaged by immunofluorescence microscopy (Figure 3). The percent co-localization of the sIgM/CD20 and LAB/CD20 pairings in light membrane vesicles was 49% and 30%, respectively. These values are in close agreement from conventional whole cell analysis suggesting that the vesicles in the light membrane fraction accurately represent the heterogeneity of plasma membrane rafts.

#### 4. Single label analysis of membrane vesicles by flow cytometry

To evaluate the utility of flow cytometry for analysis of membrane raft proteins, we first wanted to determine if light membrane vesicles could be visualised by forward scatter (FSC) and side scatter (SSC) distribution on a conventional instrument (BD FACScan) in which the only modification was a reduction in the instrument threshold. Analysis in the absence of light membranes is shown in Figure 4a (left panel). This plot shows random noise generated by electronic measurements and stray light collected by the optics system, and represents a constant background present throughout the analysis of light membranes. However, when light membranes were analyzed, a clear population of vesicles could be observed beyond background noise signals (Figure 4a, middle and right panels). To estimate the size of these

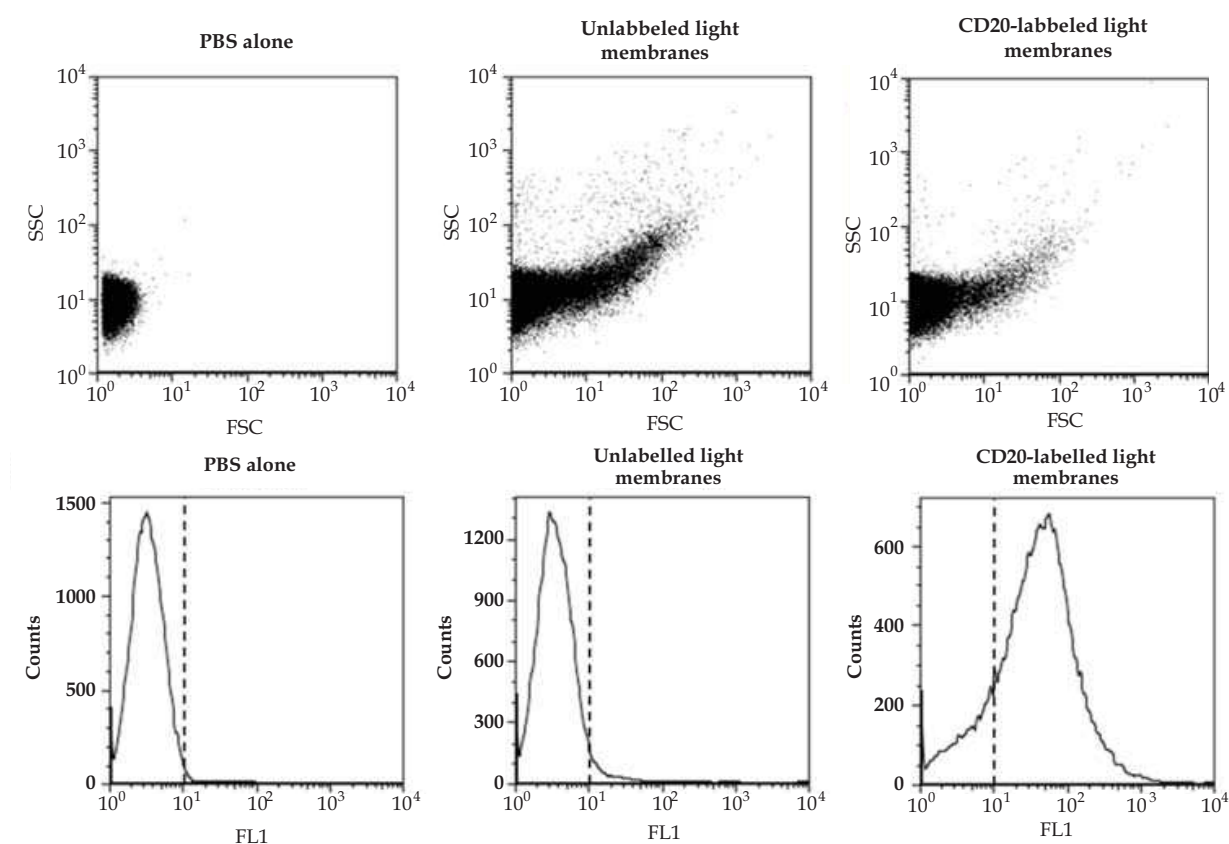


Fig. 4. Light membranes can be detected by conventional flow cytometry. Light membranes were extracted from unlabelled BJAB cells or cells labelled with anti-CD20-AF488. a) Typical FSC/SSC dot plots of the background noise (left panel) and light membranes (middle and right panels). b) Fluorescence histograms resulting from the ungated populations in (a)

vesicles, the FSC values of membrane vesicles were compared to those observed with beads of known diameter. The vesicles appeared with FSC values substantially less than the smallest bead (1  $\mu\text{m}$  diameter) (data not shown), indicating that these vesicles likely measure less than the  $1\lambda$  value and cannot accurately be measured.

We next evaluated the fluorescence profile of light membrane vesicles isolated from BJAB cells labelled with an anti-CD20-AF488 antibody. The ungated signals observed in the FSC/SSC plots in Figure 4(a) resulted in the corresponding fluorescence profiles shown in Figure 4b. Only samples isolated from cells that had been labelled with the fluorophore showed any fluorescence emission, indicating the specific detection of CD20-containing light membranes. To evaluate the smallest vesicles that could be detected by fluorescence, gates were placed at three different regions of the FSC/SSC plots with increasing FSC values, including a gate overlapping the background noise (gate setup shown in Figure 5a). As expected, fluorescence was observed in regions where light membranes were visible beyond the background noise (gates R2 and R3, Figure 5). The gated population that overlapped the background noise (R1) also generated a specific fluorescence signal, indicating that membrane vesicles were also present and detectable within this region (Figure 5). These data indicate that a further reduction in threshold would be beneficial as additional membrane vesicles are likely present, but excluded from analysis. One consequence of reducing the threshold is that background signals begin to dominate the FSC/SSC plot resulting in an underestimation of the event counts. However, a precise measurement of the number of events was not required for our analysis and, as the focus of subsequent experiments was the detection of fluorescence in a separate detection channel, a potentially saturated FSC channel was not problematic.

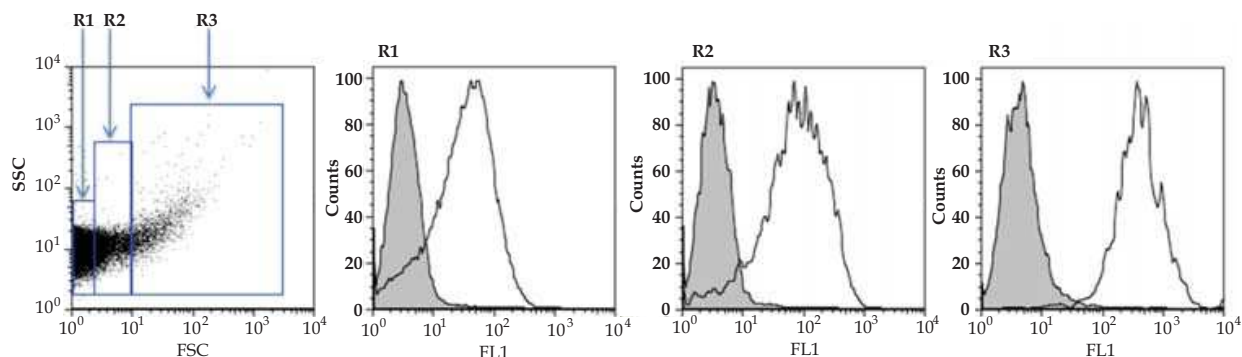


Fig. 5. Fluorescent particles can be observed in regions obscured by background noise. BJAB cells were labelled with anti-CD20-AF488. Light membranes were isolated and analyzed by flow cytometry. a) FSC/SSC plot showing gated regions R1, R2 and R3. b-d) Fluorescence histograms of R1, R2 and R3

The fluorescence histograms shown in Figures 4 and 5 indicated that the light membranes appeared to be exclusively CD20-positive with no apparent CD20-negative population. This conflicts with microscopy data which have established that a substantial fraction of CD20 occupies separate raft domains from certain other raft proteins, such as LAB and sIgM (Mutch et al., 2007, Petrie & Deans, 2002). This suggests that a CD20-negative population of light membrane vesicles were not visualized using the current method. A dilution scheme improved the resolution of the fluorescence resulting in the observation of at least two CD20 populations (Figure 6), although a distinct CD20-negative population was still not observed.

The difficulty in distinguishing distinct populations of membrane vesicles was likely the result of many membrane vesicles passing simultaneously past the light source, a consequence of the small size of membrane vesicles relative to the flow path. Dilution reduced the number of membrane vesicles detected coincidentally, thus improving the resolution at the expense of acquisition time.

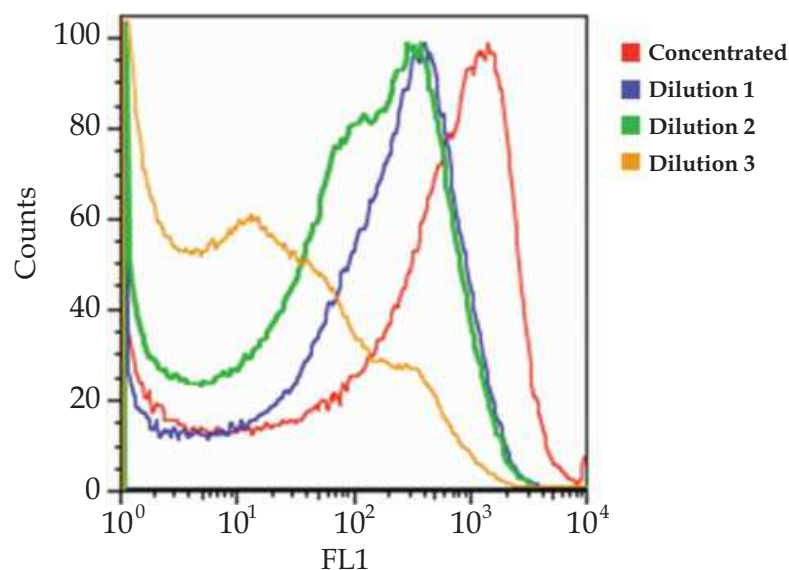


Fig. 6. CD20 heterogeneity was observed at higher sample dilutions. Light membranes obtained from anti-CD20-AF488 labelled BJAB cells were diluted 10x (dilution 1), 100x (dilution 2) and 1000x (dilution 3) from the standard volume of concentrated light membrane obtained after extraction. At greater dilutions, a bimodal distribution of CD20 fluorescence became apparent

As there was no clear CD20-negative population, we next explored the use of two fluorescence labels to determine whether flow cytometry can distinguish distinct populations of membrane raft domains.

## 5. Two-label analysis of membrane vesicles by flow cytometry

The B cell antigen receptor, or sIgM, translocates into rafts following antigen binding, and is found in a substantially distinct raft subset from CD20 (Figure 3). To analyse light membranes by 2-color flow cytometry, BJAB B cells were labelled with anti-CD20-AF488 and with anti-IgM-PE under activation conditions known to translocate IgM into rafts (Petrie & Deans, 2002, Polyak et al., 2008). The light membranes were extracted and analyzed on the BD FACScan flow cytometer with a reduced threshold. The fluorescence profile produced is shown in Figure 7, in which only small fractions of the total fluorescent membrane vesicles were uniquely sIgM-containing (sIgM<sup>+</sup>CD20<sup>-</sup>) or CD20-containing (sIgM<sup>-</sup>CD20<sup>+</sup>) (6% and 3%, respectively). The degree of association between sIgM and CD20 observed at 80% was substantially higher than reported by microscopy (Petrie & Deans, 2002, Polyak et al., 2008). This discrepancy between the two methods was attributed to an unknown factor specific to flow cytometry analysis. Therefore, modifications were made to the flow cytometry protocol in an attempt to increase the proportion of sIgM<sup>+</sup>CD20<sup>-</sup> and IgM-CD20<sup>+</sup> membrane vesicles.



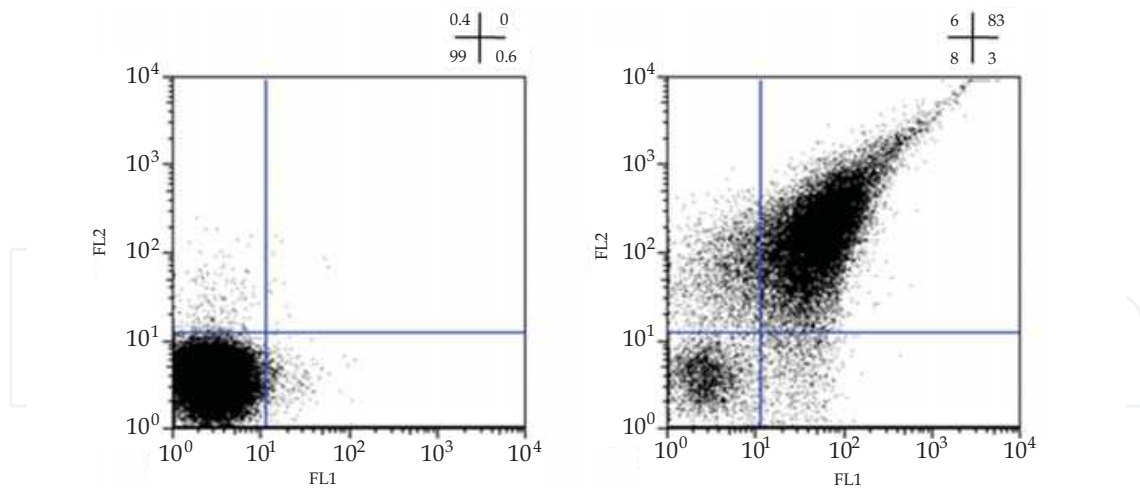


Fig. 7. Fluorescence can distinguish distinct populations of membrane raft-containing light membrane vesicles. Unlabelled BJAB B cells, or BJAB cells labelled with anti-CD20-AF488 and anti-IgM-PE were lysed and fractionated and the light membrane fractions were analysed by flow cytometry. Unlabelled light membranes showed no fluorescence (left panel) whereas fluorescence in both FL1 and FL2 channels was visible in the membranes derived from labelled cells (right panel). Populations of sIgM<sup>+</sup>CD20<sup>-</sup> and IgM-CD20<sup>+</sup> were present; however, the percentage of vesicles appearing as sIgM<sup>+</sup>CD20<sup>+</sup> was much greater than reported by microscopy

We speculated that sIgM<sup>+</sup>CD20<sup>-</sup> and IgM-CD20<sup>+</sup> vesicles would be more prevalent at decreasing size. Indeed, when vesicles with progressively smaller FSC values were analyzed, the proportion of membrane vesicles exhibiting distinct fluorescence increased (Figure 8). Thus, vesicle size, as approximated by FSC, is a major factor affecting the fluorescence distribution of membrane vesicles analyzed by flow cytometry. Subsequent analyses were performed on the 20% of vesicles appearing with the lowest FSC values.

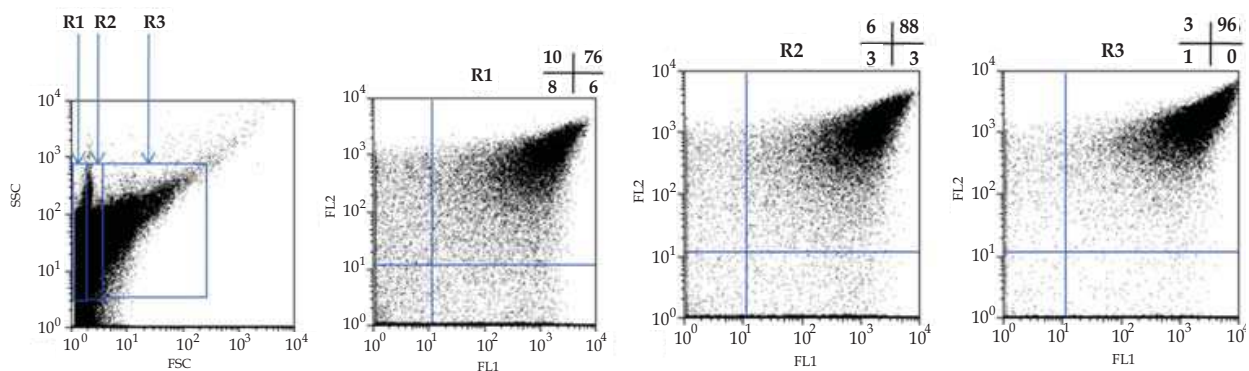


Fig. 8. Particle size influences the distribution of fluorescence. Light membranes were isolated from BJAB cells labelled with anti-CD20-AF488 and anti-IgM-PE. Gates were placed over particles with increasing FSC value (R1-R3) and the resulting fluorescence observed for the CD20<sup>+</sup>sIgM<sup>+</sup> population was 76, 88 and 96%, respectively

The specificity of the association between sIgM and CD20 within the sIgM<sup>+</sup>CD20<sup>+</sup> population was assessed by observing the fluorescence behaviour of light membranes isolated from cells that were separately labelled with either anti-sIgM-PE or anti-CD20-

AF488. The membrane vesicles isolated from each individually labelled cell group were mixed together in equal amounts immediately before flow cytometry analysis (Figure 9) and the fluorescence profiles then compared to light membranes isolated from cells that were labelled simultaneously (Figure 7). Flow analysis showed that even when cells were labelled separately, 62% of light membrane vesicles still exhibited positive staining for both markers, compared to 83% when rafts were isolated from cells labelled simultaneously. This suggests that when cells are labelled simultaneously, approximately 60% of the total sIgM<sup>+</sup>CD20<sup>+</sup> population can be attributed to non-specific association.

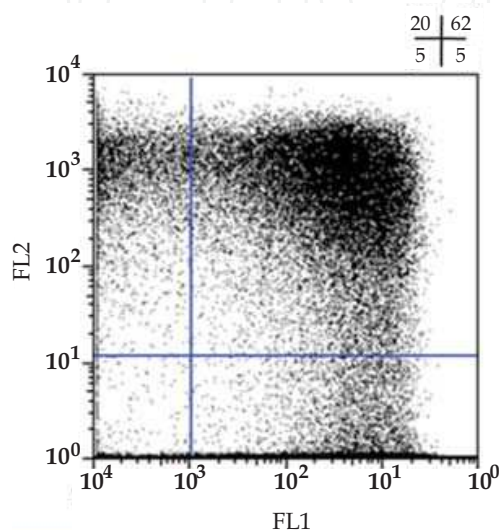


Fig. 9. Non-specific factors artifactually induce the formation of CD20<sup>+</sup>sIgM<sup>+</sup> vesicles. Separate populations of BJAB cells were labelled with either anti-CD20-AF488 or anti-IgM-PE, followed by light membrane extraction. The light membranes from each population were mixed immediately before analysis by flow cytometry. The large population (62%) of CD20<sup>+</sup>sIgM<sup>+</sup> vesicles must be derived from a non-specific association between single positive populations of vesicles

The residual 20% of the sIgM<sup>+</sup>CD20<sup>+</sup> population approximates the reported co-localization between CD20 and sIgM in microscopy studies (Petrie & Deans, 2002). Several experiments were conducted to reduce this non-specific effect, including additional mechanical disruption of the cells, adjusting the ionic strength of the solution, and sample dilutions; however, there was no improvement in the proportion of sIgM<sup>+</sup>CD20<sup>-</sup> or sIgM<sup>-</sup>CD20<sup>+</sup> single fluorescence. Despite the disproportionately large sIgM<sup>+</sup>CD20<sup>+</sup> population, there were still sufficient amounts of uniquely stained populations to evaluate flow cytometry sorting as a means to enrich the sIgM<sup>-</sup> and CD20<sup>-</sup> specific populations.

## 6. Application of flow sorting to enrich distinct sub-populations of raft containing vesicles

Flow cytometry sorting of light membranes was initially performed on BJAB cells labelled with anti-CD20-AF488. The populations selected for enrichment were the 20% most brightly labelled (CD20<sup>bright</sup>) and the 20% least brightly labelled (CD20<sup>dim</sup>) membrane vesicles (see Figure 10 for gate set-up). The sort was performed on a BD FACSVantage SE for

approximately two hours. The resulting sorted populations were re-analyzed by flow cytometry to assess if any enrichment had taken place. The sorted, CD20<sup>bright</sup> population appeared as a narrow peak (red curve shown in Figure 10). The sorted CD20<sup>dim</sup> population was represented by a broad distribution (blue curve in Figure 10) that contained fewer membrane vesicles than was observed for the CD20<sup>bright</sup> population. Overlaying the resulting fluorescence profiles of the sorted populations shows that the sorting did produce populations with different fluorescence intensities with only a small region of overlap between the two (Figure 10). To determine whether sufficient amounts of sorted material were present for mass spectrometry protein identification, the sorted populations were analyzed by SDS-PAGE (data not shown).

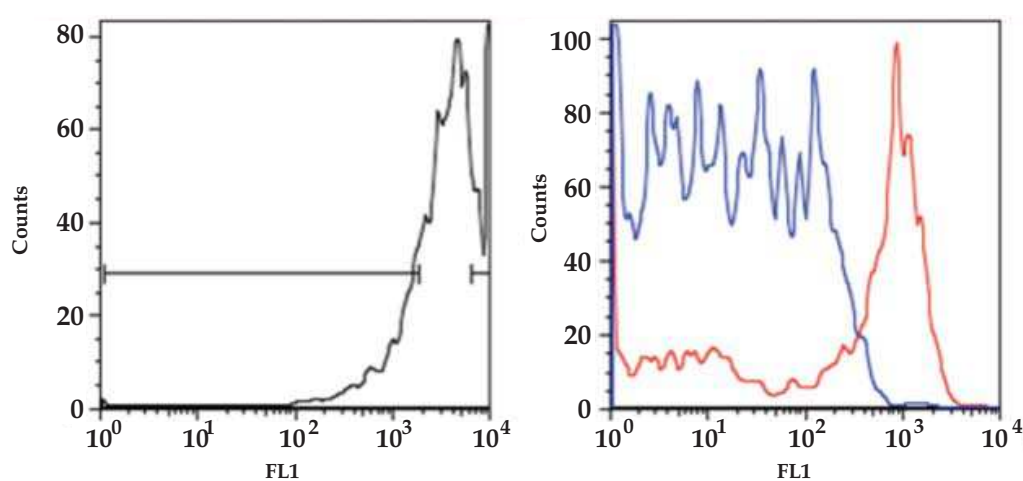


Fig. 10. Enrichment of CD20<sup>dim</sup> and CD20<sup>bright</sup> light membrane vesicles by flow cytometry sorting. BJAB cells were labelled with anti-CD20-AF488 followed by light membrane extraction. Light membranes were analyzed on a BD FACSVantage producing the fluorescence profile in the left panel. Sorting was performed on the 20% most dim and 20% brightest particles and the resulting purified populations were re-analyzed (right panel)

The CD20<sup>dim</sup>, CD20<sup>bright</sup> and a sample of unsorted material were run onto a 12.5% SDS-PAGE gel. Based on Coomassie stain intensity, the CD20<sup>bright</sup> gel lane had more protein material than the CD20<sup>dim</sup> population, which was in agreement with the poor population statistics observed in the flow cytometry sort. The staining pattern was similar across all three gel lanes. This was not unexpected given the limited sensitivity of regular Coomassie stains, and as a result no qualitative differences could be discerned based on SDS-PAGE analysis. Subsequently, the gel lanes corresponding to the CD20<sup>dim</sup> and CD20<sup>bright</sup> populations were divided into 10 gel slices, each of which was analyzed by LC-MS/MS. The number of protein identifications obtained from the CD20<sup>dim</sup> population was considerably less (approximately a third) than those from the CD20<sup>bright</sup> population (data not shown) confirming the protein concentration differences observed on the gel. CD20 was not identified in the CD20<sup>dim</sup> population, clearly a consequence of the low protein abundance. Possibly for the same reason, no proteins were uniquely identified in this population compared to the CD20<sup>bright</sup> population. Altogether, these data confirm that extracted light membranes can be sorted into distinct populations for proteomic analysis; however, greater yield from the sort is required to draw any meaningful conclusions.

Sorting of membrane vesicles isolated from BJAB cells labelled simultaneously with anti-CD20-AF488 and anti-IgM-PE, was performed on two instruments; the BD FACSVantage SE and the BD FACSAria. The compensation settings and gates on both instruments were based on vesicles with the smallest FSC value (smallest 20%), which generated the greatest proportion of CD20<sup>+</sup>IgM<sup>-</sup> and CD20<sup>-</sup>IgM<sup>+</sup> populations (shown in Figure 8b). During the compensation and gate setup, both instruments accommodated a reduced threshold; however, when the instruments began sorting, the noise contribution from the piezo-electric drive on the Vantage, and the additional electronic noise associated with either the Vantage or the Aria, prevented the reduction of the threshold to the optimal levels established during compensation. As a result, there was a dramatic reduction in the number of small vesicles responsible for generating the optimal fluorescence distribution. The gate was readjusted to include a greater proportion of vesicles with slightly larger FSC values. This modification resulted in only a minor proportion of vesicles appearing with a single fluorescent label (Figure 11, approximately 3% IgM<sup>-</sup>CD20<sup>+</sup> and approximately 1% sIgM<sup>+</sup>CD20<sup>-</sup>). The decrease in the proportion of distinct membrane vesicles would require an approximately 20-fold increase in sorting time to acquire quantities of sorted material similar to those obtained in the single label sort. This would have required an acquisition of approximately 30 hours and was not plausible to pursue further. Unfortunately, if reduced thresholds and low FSC gating are required for a desired experiment, sorting is not feasible with current instrumentation.

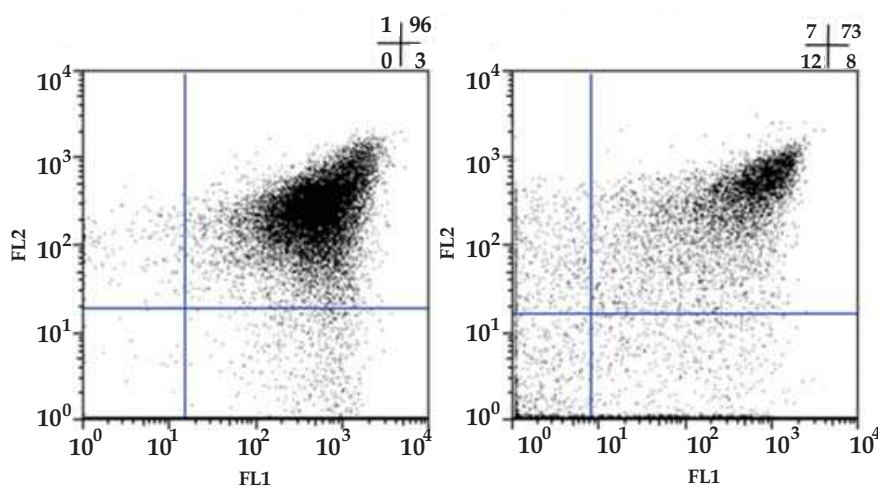


Fig. 11. Sorting particles with low FSC values results in low yields. Light membrane vesicles from BJAB cells labelled with anti-CD20-AF488 and anti-IgM-PE were analyzed on the BD FACSVantage. Initially, a low threshold setting and gate placement was possible, however when sorting was activated, the threshold increased and required gate placement on particles with higher FSC values. The result is shown in the left panel where only 3% and 1% of CD20<sup>+</sup>sIgM<sup>-</sup> and CD20<sup>-</sup>sIgM<sup>+</sup> was available. The same sample run on the BD FACScan in the absence of sorting resulted in the profile shown on the right panel

However, sorting light membranes is possible within specific experimental contexts. For instance, samples that do not require size gating, as observed with the single label identification of CD20 heterogeneity, can successfully be sorted with a conventional instrument. Little optimization would be required to sort sufficient CD20<sup>dim</sup> and CD20<sup>bright</sup>



populations for biochemical analysis. In addition, this type of enrichment could be used for other populations of membrane raft proteins with heterogeneity observed by flow cytometry, such as GM1, as presented by Morales-Garcia et al. (Morales-Garcia et al., 2008). Even in the absence of sorting, the flow cytometry detection of membrane raft proteins could become a valuable diagnostic tool, as this method can identify heterogeneity imperceptible in conventional whole cell flow analysis.

## **7. Future requirements for successful flow sorting of small membrane vesicles**

The threshold settings of the flow cytometer had to be reduced in order to detect smaller vesicles; however, there are still inherent limitations to using an instrument designed for cells approximately an order of magnitude larger than the membrane vesicles under investigation. The development of micro-flow cytometry systems could address many of the obstacles encountered in membrane raft analysis. The most important instrumental developments are related to the fluidics path, where poorly focused membrane vesicles will be most problematic. The use of a high flow rate (60  $\mu\text{l}/\text{min}$ ) was employed to minimize the central core generated by the sheath fluid. However, the substantial improvement of CD20 heterogeneity resolution at higher dilutions suggested that the diameter of the central core, even at 60  $\mu\text{l}/\text{min}$ , was too large relative to the size of the vesicles. This large central core probably could not focus membrane vesicles into single file, resulting in membrane vesicles passing the laser coincidentally. The dilution of the membrane raft suspension improved the appearance of the heterogeneous populations by reducing the frequency of these coincident events at the expense of longer acquisition times. Focusing membrane vesicles into a smaller central core may not be possible using hydrodynamic focusing (Ateya et al., 2008), but recent developments in alternative focusing techniques can be applied on a scale amenable to micro-flow cytometry. The most relevant development to flow cytometry is microfluidic inertial focusing (Oakey et al., 2010). This technique exploits inertial fluidic forces within microfluidic channels to precisely position particles and has been used as a replacement for the traditional hydrodynamic focusing used in most conventional flow cytometry instruments (Oakey et al., 2010).

Another critical location of the flow path within a flow cytometer is the sorting interface. The electrostatic sorting commonly used in flow cytometry involves passing a focused cell or vesicle suspension into a vibrating nozzle. This causes the stream to break into highly uniform droplets, which are then deflected into collection tubes through the application of a voltage at precisely timed intervals. The nozzle size dictates the size of the droplet generated by this process and the recommendation for high purity sorting is that the size of the cell (or membrane vesicle in this case) should be at least 20-25% the size of the nozzle orifice (Macey, 2007). The 70  $\mu\text{m}$  nozzle designed for 5-10  $\mu\text{m}$  cells was the smallest available nozzle for the instruments used in this study. The membrane vesicles under observation were less than 1  $\mu\text{m}$ , which would correspond to an ideal nozzle size of 5  $\mu\text{m}$ . It is likely that sorting with the 70  $\mu\text{m}$  nozzle would have made sorting high purity populations even more challenging, since at this size the drops formed would likely contain more than a single membrane vesicle. This is especially true since these experiments required the raft suspension to be in a small volume resulting in vesicles in close spatial proximity.



Miniaturization of the nozzle would greatly improve the effectiveness of sorting membrane vesicles by flow cytometry and is currently in development (Ateya et al., 2008). Flow cytometry sorting remains an excellent opportunity to isolate and fully characterize the protein heterogeneity in membrane raft subsets. Developments in miniaturizing flow cytometry systems are essential to sort smaller vesicles effectively.

## 8. Conclusion

Flow cytometry was investigated as a means to characterize proteins present in plasma membrane rafts, isolated from labelled cells as light membrane vesicles. Vesicles obtained from BJAB cells labelled with an anti-CD20 fluorescent antibody revealed heterogeneously stained populations when analysed by flow cytometry. This was used as a model system to investigate whether sorting could be employed to isolate two populations of membrane vesicles, CD20<sup>dim</sup> and CD20<sup>bright</sup>. Sorting was successful, as enrichment of dim and bright fluorescence was observed on the sorted populations. Subsequent biochemical analysis was limited as the amount of protein material obtained from the sort was low. Longer sorting acquisition times would be necessary to acquire sufficient material for meaningful characterization. Flow cytometry was also used to identify membrane vesicles with distinct sIgM- and CD20-containing populations. Detection of the largest proportion of sIgM<sup>+</sup>CD20<sup>-</sup> and sIgM<sup>-</sup>CD20<sup>+</sup> populations required a size gate in which the smallest 20% of membrane vesicles, as determined by FSC, were selected. A reduced threshold was also required to permit the analysis of even smaller membrane vesicles. Unfortunately, enrichment of the sIgM<sup>+</sup>CD20<sup>-</sup> and sIgM<sup>-</sup>CD20<sup>+</sup> populations by sorting could not be performed due to the automated adjustment of instrumental threshold. This substantially reduced the proportion of membrane vesicles available for sorting and the time necessary to collect sufficient protein material for further biochemical analysis became prohibitive.

Future developments in miniaturization of flow cytometry systems (fluidics and nozzle sizes) are essential for this type of flow cytometry analysis. As the technological developments advance, it may become possible to fully characterize membrane raft domains or other cellular sub-domains that cannot be extensively studied due to limited isolation schemes.

## 9. Acknowledgments

The authors wish to thank Laurie Kennedy and Laurie Robertson for their assistance with sorting at the University of Calgary Flow Cytometry Core Facility. Also thanks to Wei-Xiang Dong for his assistance in collecting the TEM images at the Microscopy & Imaging Facility at the University of Calgary.

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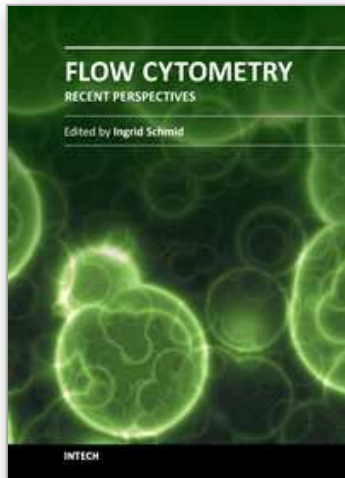
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Edited by M.Sc. Ingrid Schmid

ISBN 978-953-51-0626-5

Hard cover, 500 pages

**Publisher** InTech

**Published online** 13, June, 2012

**Published in print edition** June, 2012

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