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Use of SYTO 13, a fluorescent dye binding nucleic acids, for the detection of microparticles in *in vitro* systems

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Abstract

Microparticles (MPs) are small membrane-bound vesicles that are released from activated or dying cells by a blebbing process. These particles contain nuclear and cytoplasmic components and represent unique biomarkers for disease. The small size of particles, however, limits detection using flow cytometry with either light scatter or staining for surface markers. Because MPs contain DNA and RNA, we have explored the use of SYTO 13, a member of the class of SYTO dyes, for particle detection. SYTO 13 is cell permeable and has a high fluorescent yield when bound to DNA or RNA. In this study, we compared detection of MPs using either light scatter or SYTO 13 staining, testing the hypothesis that, with fluorescence detection with SYTO 13, problems of “noise” with light scatter are reduced and the range of MP sizes detected is increased. In these experiments, particles were obtained from lymphoid cell lines treated *in vitro* to undergo apoptosis. As these results showed, STYO 13 allowed the detection of 1.5 to 2.9 times as many particles as did light scatter. The increased sensitivity was observed with 3 different cell lines and was independent of inducing stimulus. Treatment of fixed and permeabilized MPs with DNase and RNase showed that SYTO 13 binding resulted from interaction with both DNA and RNA. Together, these findings indicate that the nucleic acid content of MPs provides the basis for their detection in *in vitro* systems and suggests the utility of fluorescent dyes like SYTO 13 for more sensitive quantitative assays.

Keywords

SYTO 13; nucleic acid dye; microparticles; apoptosis; DNA; RNA

Introduction

Microparticles (MPs) are small membrane-bound vesicles that can serve as signaling elements to mediate intercellular communication in physiological and pathological settings. These particles are released from activated and dying cells by a blebbing process and contain both nuclear and cytoplasmic components. Once in the extracellular milieu, microparticles can display potent biological activities that impact on inflammation and thrombosis among other processes. Given their origin in cell activation and cell death, microparticles can show increased numbers in diseases such as atherosclerosis, sepsis, and rheumatoid arthritis among many others (1-5).

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In general, the measurement of microparticles involves flow cytometric techniques. With this method, microparticles show a size distribution of 0.1 to 1.0 μm in diameter and are thus smaller than cells or apoptotic bodies. Apoptotic bodies represent large remnants of dying cells as well as their collapsed remains and can be 2-3 μm or greater in diameter (1,6). In biological fluids or media from cultured cells, microparticles can be measured on the basis of light scattering, frequently using preparations in which cells and other larger material have been removed by centrifugation. Microparticles can also be identified on the basis of cell surface markers such as annexin V as a general indicator of cell death or specific markers (e.g., CD61 and CD41 for platelets) for particular cell populations (1,6-8).

While flow cytometry has been the standard for microparticle assay, this technique has inherent limitations. Thus, with light scattering, establishing appropriate windows for detection can be difficult since most instruments are not designed to measure objects of this size range, with particles overlapping “noise” (4,9). Similarly, since particles are small and variably display membrane markers, the signals arising from the binding of annexin V or antibodies to cell surface markers can be low (e.g., hundreds to thousands of times less than a cell). Although annexin V staining has been commonly used to enumerate microparticles because of the surface phosphatidylserine exposed during apoptosis, many particles fail to bind this molecule in detectable amounts (6,10,11). Thus, flow cytometry may fail to measure accurately the number of particles in a sample whether the method is based on size, expression of cell surface markers or a combination of these methods.

In this paper, we have explored a new flow cytometric approach for the measurement of particles based on the use of a nucleic acid binding dye. Among their components, microparticles contain both DNA and RNA. In recent studies, we showed that microparticles generated *in vitro* from apoptotic cell lines contain both DNA and RNA, including ribosomal RNA, messenger RNA and low molecular weight species consistent with microRNA. Furthermore, using both fixed and unfixed microparticle preparations, we demonstrated particle detection by flow cytometry using staining with propidium iodide (12).

In the current studies, we have extended these observations to develop an assay for the detection of microparticles generated from cell lines *in vitro*. Adapting an approach to measure bacteria by flow cytometry (13-16), this assay is based on the use of cell permeant dye, SYTO 13, which binds both DNA and RNA. The use of SYTO 13 eliminates the need for fixation to detect internal nucleic acids. As results presented herein indicate, because of the high fluorescence yield of SYTO 13, its use in flow cytometry leads to a significant increase in the detection of microparticles generated *in vitro* from cell lines. Given the simplicity of this technique and its application with ordinary cytometers, the use of nucleic acid binding dyes should help elucidate microparticle release and their role as markers of important biological processes *in vivo* and *in vitro*.

Materials and Methods

Cell culture and treatment

Jurkat T cell leukemia cells, HL-60 promyelocytic leukemia cells and MOLT-4 T-lymphoblasts were obtained from the Duke University Comprehensive Cancer Center Cell Culture Facility and were grown in Complete Medium (CM). CM was prepared by supplementing RPMI 1640 (Gibco BRL, Carlsbad, CA) with 10% heat inactivated fetal bovine serum (FBS) (Hyclone, Logan, UT) and 20 μg/ml gentamicin (Gibco). Cells in the logarithmic growth phase were harvested and pelleted by centrifugation at 400 × g for 5 min. The cell pellet was then resuspended at a density of 2.5×10^6 cells/ml in CM and cultured at 37°C in 5% CO_2. To induce apoptosis and microparticle generation, 10^7 cells

were treated with either 10 μM etoposide (ETO), 10 μg/ml camptothecin (CAMP) or 1 μM staurosporine (STS) (all from Sigma-Aldrich Co., St. Louis, MO) in a 6-well tissue culture plate (Cellstar #657-160, Greiner bio-one, Monroe, NC). Treated cells were incubated for 18 hr. The media were collected and used for analysis of MPs by flow cytometry as described below.

Isolation and preparation of microparticles

MPs were prepared for counting by three methods. In the first method, the treated cell suspension was centrifuged at 400 × g for 5 mins at room temperature to pellet out the cells. The cell-free supernatant containing MPs was aspirated into fresh microfuge tubes and directly used for flow cytometric analysis. The second method for preparing MPs used a two-step differential centrifugation process, also at room temperature. The treated cell suspension was first centrifuged at 400 × g for 5 min to remove cells as described. Next, 1 ml aliquots of the supernatant were centrifuged at 16,000 × g for 30 min in a microcentrifuge (Denville 2600, Denville Scientific, Inc., Metuchen, NJ) to prepare the MP pellet. MP pellets were resuspended in 1 ml of calcium (Ca^{++}) and magnesium (Mg^{++}) free phosphate buffered saline (PBS) (Gibco BRL) to bring it to the original volume of the treated cell culture that was centrifuged.

In the third method for MP preparation, the treated cell culture was first centrifuged at 400 × g for 5 mins to pellet out the cells. The supernatant containing MPs was then aspirated into a 5 ml syringe (BD Biosciences, San Jose, CA) and filtered through either 0.45 μm, 0.22 μm or 0.1 μm PVDF or PES syringe filters (Millipore, Carrigtwohill, Co. Cork, Ireland). The MPs were then quantified and characterized by flow cytometry analysis. All three methods of isolation produced similar results.

Flow cytometric analysis

MPs were counted and analyzed on a BD FACScan flow cytometer equipped with a 15 mW, 488 nm, air-cooled argon-ion laser and three photomultipliers with band pass filters of 530 nm (FL1), 585 nm (FL2) and >650 nm (FL3). Data were acquired using BD CellQuest PRO software (BD Biosciences). A fifty μl aliquot of the MP suspension was diluted to 400 μl (1:8) in PBS or in 200 nM solution of SYTO 13 (Molecular Probes, Inc., Eugene, OR). The 200 nM SYTO 13 solution was prepared by diluting 1 μl of 5 mM SYTO 13 in 25 ml of PBS. The MP suspension was prepared as described above at the same concentration as in the original cell culture. For MP size determination, control latex microspheres of sizes 0.1 μm, 0.5 μm and 1.0 μm (Fluorsbrite™ Plain YG microspheres, Polysciences, Inc., Warrington, PA) were analyzed by flow cytometry using both fluorescence and light scatter. Data were collected in logarithmic mode at all channels. Analysis was carried out using FlowJo flow cytometry analysis software (Tree Star, Inc., Ashland, OR).

To establish optimal instrument settings to exclude background noise, PBS alone or 200 nM SYTO 13 alone was used as a blank to set a side scatter threshold or FL-1 threshold, respectively. For this purpose, the blank was first titrated at SSC photomultiplier tube (PMT) voltages ranging from 100 – 900 volts with corresponding threshold values ranging from 0 – 800 and adjusted in increments of 25, for each PMT voltage setting. Those voltage-threshold combinations were noted at which background noise was reduced or eliminated. A similar titration was performed for PMT voltages ranging from 100 – 900 volts for the FL-1 channel using 200 nM SYTO 13 as a blank.

A suspension of MPs was prepared as described above and diluted 1:10, 1:50 and 1:250 in either PBS or 200 nM SYTO 13. These MP dilutions were analyzed as done for the blanks over a range of PMT voltage and threshold values. The combination of PMT voltage and

threshold value for SSC or FL-1 that produced the highest counts at all MP concentrations with minimal (<0.05% of total counts) background "noise" was set as the optimum for all further experimental observations. Therefore, the SSC PMT was set to 300 volts with the threshold set at 175 on this machine. The FL-1 PMT was set at 800 volts with a threshold at 350. All observations were made in log mode.

To determine MP numbers in the cell culture, the sample flow rate was first calibrated by weighing sample tubes before and after each 10-min run and averaging the differences. This sample flow rate was multiplied by the amount of time taken for each measurement to determine the total volume sampled. The final MP concentration was calculated by dividing the total counts measured by the total volume sampled and further by multiplying by the dilution factors used in preparing the sample solution loaded on the flow cytometer. To reduce the effect of dead volume, data acquisition was started 6 to 8 seconds after introducing the sample into the system and stabilization of detection. A two-tailed t-test for unpaired samples with unequal variances was used for statistical analysis of MP numbers.

Nuclease digestion of nucleic acids in MPs

To assess staining of nucleic acids by SYTO 13 in MPs, cells were either fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD Biosciences, San Jose, CA) as per the manufacturer's instructions and then treated with nucleases, or treated with nucleases without permeabilization. Undigested, control MPs were similarly either fixed and permeabilized or left untreated. Briefly, the MP pellet isolated from 2.5×10^6 Jurkat cells was resuspended in 250 μl of BD Cytofix/Cytoperm™ solution in a microfuge tube and incubated at 4°C for 20 min. MPs were then pelleted at 16,000×g for 30 min and washed twice in 500 μl of the BD Perm/Wash™ buffer containing a permeabilizing agent (saponin) and resuspended in 200 μl of the same buffer. This volume of the enriched and permeabilized MP suspension was treated with 100 U/ml of RNase-free DNase (Invitrogen Co., Carlsbad, CA) or 200 U/ml of DNase-free RNase (Sigma-Aldrich Co.) or both and incubated at 37°C for 90 min and then assessed for the presence of nucleic acid. The final volume of the treated MP suspension was brought up to 1 ml by adding more BD Perm/Wash™ buffer. The undigested, control MPs were analyzed using flow cytometry after staining with PI.

Results

Comparison of MP detection by light scatter or SYTO 13 staining

In these experiments, we compared the detection of MPs by either light scatter or staining with SYTO 13. Since MPs contain both DNA and RNA, we tested the hypothesis that a fluorescence assay would enhance particle detection and reduce potential difficulties from establishing appropriate windows by light scatter. This approach resembles detection of bacteria by fluorescence using SYTO dyes (15-19) and depends on an adequate concentration of either DNA or RNA inside the particle.

As a source of MPs, we used preparations from Jurkat T leukemia, HL-60 promyelocytic leukemia and the MOLT-4 T-lymphoblast cell lines treated for 18 hours with agents to induce apoptosis. We then determined MP numbers in the cell free supernatant by flow cytometry using side scatter (SSC) and compared them to MP numbers obtained using fluorescence in the FL-1 channel from a cell free supernatant stained with SYTO 13. Thresholds were set in both the SSC and FL-1 channels, using a PBS solution or 200 nM SYTO 13 as blanks to exclude background "noise;" to establish optimum settings, we performed an extensive analysis varying detector voltages and threshold values as described in Materials and Methods.

Figure 1 shows the comparison of MP counts measured by side scatter or fluorescence from the different cell lines induced to undergo apoptosis and MP production by treatment with 1 μM STS, 10 μM ETO or 10 μg/ml CAMP. In all cases, the MP numbers measured by fluorescence using SYTO 13 staining exceeded those counted by SSC. The ratio of SYTO 13 counts to SSC counts varied depending on cell type as well as treatment. In these experiments, staining with SYTO 13 led to the detection of 1.5 to 2.9 times as many particles as did light scatter.

In the size range for MPs, establishing thresholds for detection can be difficult using conventional flow cytometers which have been developed for cells which are much larger. In contrast, staining with SYTO 13 is based on fluorescence and, as such, facilitates event detection and separation from background. We therefore investigated the relationship of particle detection by light scatter and SYTO 13 staining. To determine whether the higher SYTO 13 counts were inclusive of all MPs detected by SSC, we used the cell free supernatants stained with SYTO 13 from Jurkat cells treated with STS, CAMP or ETO, and counted particles using both FL-1 as well as SSC detection.

As expected, there was a significant overlap of MPs counted using SSC detection and those detected by fluorescence (Fig. 2A-C; quadrant Q2). A proportion of the total counts detected by SSC, ranging from 3% -30%, were not detected by FL-1 (Fig. 2A-C; quadrant Q3). Furthermore, a distinct population of MPs was detected using fluorescence in the FL-1 channel as compared to detection in the SSC channel. The side scatter profile of MPs detected by FL-1 shows that 50% - 72% of the total events counted lie within a size range that is not seen when detected using SSC (Fig. 2A-C; quadrant Q1).

Size range of particles detected by SYTO 13 staining

In addition to MPs, cells can release other particles such as exosomes which originate from the multivesicular body and show a much smaller size distribution than MPs (20,21). Since exosomes (as well as mitochondria and chromatin fragments) also contain nucleic acid (22,23), we performed further experiments to demonstrate that the particles detected by staining with SYTO 13 were MPs as defined by their size. To show that particles staining with SYTO 13 were in the size range of MPs, we measured particle numbers in the cell free supernatants filtered through syringe filters of pore sizes ranging from 0.45 μm to 0.1 μm. As data presented in Figure 3 indicate, filtration of supernatants through the 0.1 μm filter abrogated counts by both fluorescence as well as SSC.

As these findings also indicate, filtration of the supernatants through the 0.22 μm filter abrogated MP counts detected by SSC indicating that this detection parameter is limited to MP sizes greater than 0.22 μm. This finding differs from that with SYTO 13 which still detected particles in the filtrate from the 0.22μm filter. Thus, FL-1 detection offers greater sensitivity with a significant number of events detected in the size range between 0.22 μm to 0.1 μm (Fig. 3).

The results of the filtration assays were confirmed by a comparison of particles with fluorescent beads of defined size. As shown in Figure 4, MPs detected using side scatter thresholds appear as a narrow peak (grey peak) compared to MPs detected using fluorescence thresholds (black peak). In this experiment, a greater proportion of MPs is observed in the 0.1 μm to 0.5 μm size range by fluorescence compared to side scatter as demonstrated by the left shift of the peak obtained from SYTO 13 stained MPs (black peak). Together, these findings indicate that particles detected by SYTO 13 are in the size range of MPs; however, they do not exclude the possibility that, for other cell lines or treatments, exosomes that stain with SYTO 13 could be detected.

Nucleic acid staining of MPs by SYTO 13

SYTO 13 binds strongly to nucleic acids and has extremely low intrinsic fluorescence (quantum yield <0.01) that increases significantly (quantum yield >0.4) when bound to nucleic acids (24). The spun supernatant samples were, therefore, treated with nucleases to confirm that SYTO 13 stained the nucleic acids in MPs, which in turn was detected in the FL-1 channel. The MPs derived from STS treated Jurkat and HL-60 cells were first permeabilized to allow the nucleases access to the nucleic acids within.

As results of these experiments indicate, RNase and DNase treatment of permeabilized MPs had different effects on MP counts by FL-1 detection from Jurkat and HL-60 cells. The MP counts from Jurkat samples treated individually with RNase or DNase were similar showing comparable reduction in total counts (Fig. 5, top left panel). However, DNase treatment caused a greater reduction in MP counts from the HL-60 samples than did RNase treatment (Fig. 5, top right panel). The combined action of both nucleases caused the greatest reduction in total MP counts detected by FL-1 in both cell types (Fig. 5, top panels).

Another result of these experiments concerns the requirement for permeabilization for nuclease action. Thus, as shown in Figure 5, MPs that were not permeabilized prior to nuclease treatment did not show any significant changes in MP counts by either FL-1 detection or SSC detection (bottom panels). This finding suggests that the DNA or RNA bound by SYTO 13 is in the interior of the particle or otherwise not readily accessible to the nucleases.

In contrast to its effect on measurement of MPs by fluorescence, nuclease treatment did not significantly alter the MP counts obtained by SSC detection (Fig. 5). Together, these findings indicate that MP detection by SYTO 13 results from binding of both DNA and RNA that is not directly accessible to nuclease and may vary in amounts depending on the cell of origin.

Discussion

Results presented herein demonstrate that the nucleic acid-binding dye SYTO 13 can provide a sensitive and quantitative means for detecting microparticles in *in vitro* systems. Thus, with particles obtained from apoptotic cell lines, SYTO 13 staining increased detection of particles as compared to measurement with light scattering. As a method of particle detection based on fluorescence rather than size, staining with SYTO 13 is technically simpler and more reliable since it does not involve the limitation in setting windows for light scatter measurements.

The SYTO family of dyes has been extensively studied with both eukaryotic and prokaryotic cells (13,15,16,25-27). These dyes are cell permeant and readily enter living cells to bind both DNA and RNA. Since they are non-toxic, SYTO dyes allow measurement of events such as apoptosis over time and do not require use of fixatives. Importantly, these dyes interact with both DNA and RNA in the cell although the extent may vary depending on the particular dye (19,27,28). As a result, the same dye is useful irrespective of the concentration of constituent nucleic acids in the particles.

As shown in studies of *in vitro* cell systems, SYTO dyes can yield a lower fluorescent signal with apoptotic compared to live cells (29-31). This effect may relate to changes in the structure of chromatin during apoptosis that may alter dye interaction. Furthermore, during apoptosis, both DNA and RNA are degraded, potentially decreasing dye binding (25,27,29). Since the particles we have studied are derived from apoptotic cells, their nucleic acids may

have the same reduced interaction with SYTO 13 as does the apoptotic cell. This reduction does not affect this method, however, since the overall fluorescent signal is high.

The difference in the number of particles measured with light scattering and SYTO 13 is notable. Thus, with particles from apoptotic Jurkat cells, SYTO 13 staining led to the detection of as many as 3-4 times more particles than does light scattering. This result reflects the inherent limitations of flow cytometry in measuring very small structures by light scattering. Given performance characteristics of available instruments, it is likely that light scattering fails to detect many of the smaller particles because of their overlap with "noise" (9,32). When studying events *in vitro*, the advantages of staining with SYTO 13 are considerable as this approach allows the detection of particles of a broader range of sizes.

In this regard, while SYTO 13 can interact with both RNA and DNA, the molecule bound may differ depending on cell type. Interestingly, in the use of nucleic acid dyes for staining for bacteria, the RNA in certain species does not seem to bind SYTO 13 effectively (15). As our studies indicated, with microparticles from the cell lines, both DNase and RNase treatment reduced binding although, without another way to quantify the content of these molecules, we cannot be certain the binding to DNA and RNA is equivalent in the different cell types. In this regard, since some particles may lack DNA or RNA, light scatter nevertheless represents a valuable method for particle detection especially when used in concert with SYTO13.

As suggested by our experiments, the use of SYTO 13 to measure particles should simplify the measurement of particles. In addition to increasing particle detection, the use of SYTO 13 staining allows assessment of the nucleic acid content or possible changes once the particles have been released from cells into the extracellular space. These issues may be important when using blood and other biological fluids since those particles present may be subject to nuclease digestion or arise from cells (e.g., platelets) that lack nuclei and therefore may be low in DNA. Studies are therefore in progress to elucidate further the use of SYTO 13 for measuring particles in *in vitro* and *in vivo* systems

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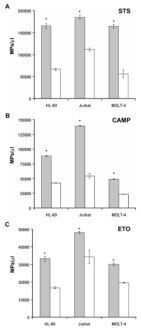

Figure 1. Comparison of MP counts detected by SYTO 13 staining and SSC

HL-60, Jurkat and MOLT-4 cells (10^7 cells) were treated with 1 μM STS (A), 10 μg/ml CAMP (B) or 10 μM ETO (C) and supernatants harvested 24 hours later for analysis. The MPs produced by these cells were detected in the cell free supernatants using fluorescence due to SYTO 13 staining (shaded bars) or using SSC (clear bars) in unstained samples. MP counts by SYTO 13 detection or SSC detection were analyzed and were significantly different. * = $p<0.0004$ in all cases.

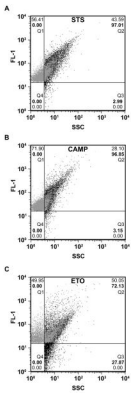

Figure 2. MP populations detected by SSC and STYTO 13 staining

Cell free supernatants containing MPs from Jurkat cells treated with 1 μM STS (A), 10 μg/ml CAMP (B) or 10 μM ETO (C) was stained with 200 nM SYTO 13. The dot-plots show MPs detected by SSC (black dots) or fluorescence (grey dots). Numbers in bold face denote percentage of MPs detected by SSC in each of the quadrants (Q1 – Q4), and numbers in regular font indicate percentage of MPs detected by fluorescence. The populations of MPs detected by both SSC and fluorescence appear in quadrant Q2.

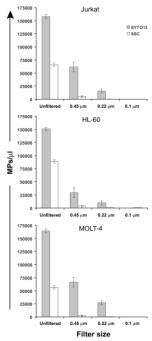

Figure 3. Size range of MPs detected by SSC or SYTO 13 staining

Jurkat (top), HL-60 (center) and MOLT-4 (bottom) cells were treated with 1 μM STS for 18 hr. The cell free supernatants with the resulting MPs was filtered through 0.45 μm, 0.22 μm and 0.1 μm syringe filters to determine the size range of MPs in suspension. MPs were counted by fluorescence from SYTO 13 staining (shaded bars) or by SSC (clear bars).

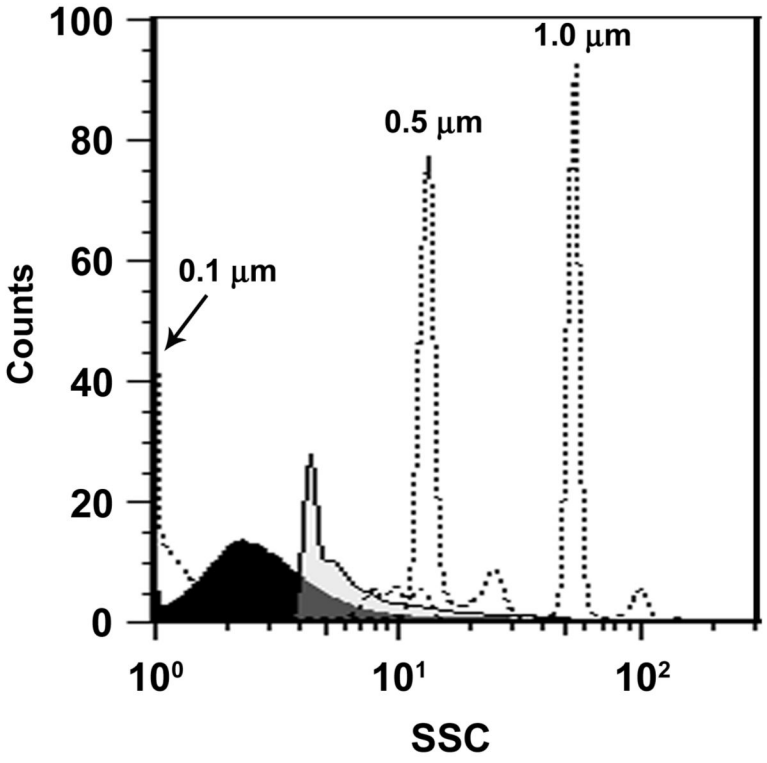

Figure 4. Determination of MP size based on control microspheres of known size

Jurkat cells were induced to undergo apoptosis by treatment with 1 μM STS for 18 hr. The resulting MPs were detected using fluorescence (black peak) or side scatter (grey peak). The histograms for MPs were compared with the histograms for control latex microspheres/ beads (dotted lines) of known sizes (0.1 μm, 0.5 μm & 1.0 μm) collected by fluorescence. The data for the beads detected by fluorescence are presented for side scatter. By side scatter detection, the 0.1 μm beads appear as a broad distribution that is poorly resolved.

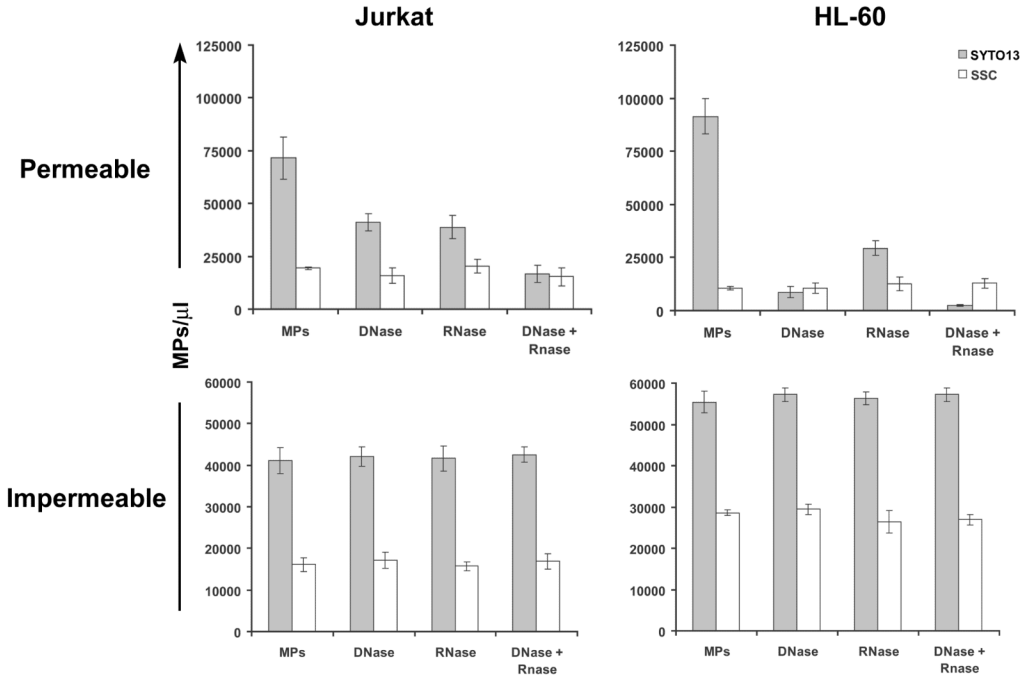

Figure 5. The effects of nuclease digestion on MP detection

Jurkat (left column) and HL-60 (right column) MPs were tested for the effect of nuclease digestion on SYTO 13 staining and subsequent MP detection by fluorescence. The cells were treated with 1 μM STS for 18 hr and the resulting MPs were permeabilized (top row) or left untreated (bottom row). MPs were then treated with DNase, RNase or both for 90 min at 37°C. Untreated MPs were used as controls. The MP counts in suspension were detected by fluorescence (shaded bars) or SSC (clear bars).