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Flow Cytometry Concepts and Capabilities to Assess Environmental Contaminants

This scientific literature summary of recent ways that flow cytometry has been used to assess the impact of environmental contaminants was compiled by Becky Hoffman of the WSLH Flow Cytometry Unit in July 2010. **For information on the WSLH Flow Cytometry Unit's capabilities, please contact Ms. Hoffman at beckyh@mail.slh.wisc.edu or 608-224-6260**.

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Cytometry July 2010 77:7(677-685)

Detection of TiO2 by flow cytometry

Zucker RM, Massaro EJ, Sanders KM, Degn LL, Boyes WK.

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Evaluation of the potential hazard of man-made nanomaterials has been hampered by a limited ability to observe and measure nanoparticles in cells. In this study, different concentrations of TiO(2) nanoparticles were suspended in cell culture medium. The suspension was then sonicated and characterized by dynamic light scattering and microscopy. Cultured human-derived retinal pigment epithelial cells (ARPE-19) were incubated with TiO(2) nanoparticles at 0, 0.1, 0.3, 1, 3, 10, and 30 microg/ml for 24 hours. **Cellular reactions to nanoparticles were evaluated using flow cytometry and dark field microscopy**. A FACSCalibur flow cytometer was used to measure changes in light scatter after nanoparticle incubation. Both the side scatter and forward scatter changed substantially in response to the TiO(2). From 0.1 to 30 microg/ml TiO(2), the side scatter increased sequentially while the forward scatter decreased, presumably due to substantial light reflection by the TiO(2) particles. Based on the parameters of morphology and the calcein-AM/propidium iodide viability assay, TiO(2) concentrations below 30 microg/ml TiO(2) caused minimal cytotoxicity. Microscopic analysis was done on the same cells using an E-800 Nikon microscope containing a xenon light source and special dark field objectives. At the lowest concentrations of TiO(2) (0.1-0.3 microg/ml), the flow cytometer could detect as few as 5-10 nanoparticles per cell due to intense light scattering by TiO(2). Rings of concentrated nanoparticles were observed around the nuclei in the vicinity of the endoplasmic reticulum at higher concentrations. These data suggest that the uptake of nanoparticles within cells can be monitored with flow cytometry and confirmed by dark field microscopy. This approach may help fulfill a critical need for the scientific community to assess the relationship between nanoparticle dose and cellular toxicity. **Such experiments could potentially be performed more quickly and easily using the flow cytometer to measure both nanoparticle uptake and cellular health.**

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Inhalation Toxicology**,** Volume 19, Issue S1 2007, pages 59 - 65 DOI: 10.1080/08958370701493282

Cellular Toxicity of Various Inhalable Metal Nanoparticles on Human Alveolar Epithelial Cells

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Nanoparticles (NPs) have a greater potential to travel through an organism via inhalation than any other larger particles, and could be more toxic due to their larger surface area and specific structural/chemical properties. The aim of this study was to evaluate in vitro biological effects of various inhalable metallic NPs (TiO_2, Ag, Al, Zn, Ni). Human alveolar epithelial cells (A549) were exposed to various concentrations of NPs for 24 h. The extent of morphological damage was in the order of m-TiO_2 > n-TiO_2 > m-silica >> n-Ni ≈ n-Zn ≈ n-Ag ≈ n-Al and was affected in a dose-dependent manner. **The extent of apoptotic damage measured with two-color flow cytometry was in the order of n-Zn > n- Ni > m-silica >> n- TiO_2 > m-TiO_2 > n-Al > n-Ag**. The extent of apoptotic damage measured with DNA fragmentation was in the order of n-Zn ≈ m-silica > n- Ni >> m- TiO_2 ≈ n- TiO_2 ≈ n-Al > n-Ag, indicating no significant difference in the damages by both m-TiO_2 and n-TiO_2. The extents of apoptotic damages were also affected in a dose-dependent manner. Uptake of no other NPs but n-TiO_2 and m-TiO_2 into the cells was observed after 24 h exposure. The intracellular generation of ROS was significant with n-Zn but not with the other particles. These results demonstrated that various inhalable metallic NPs (TiO_2, Ag, Al, Zn, Ni) could cause cell damages directly or indirectly. More detailed studies on the influence of size, structure, and composition of the NPs are needed to better understand their toxic mechanisms.

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Environ. Sci. Technol. **2007,** 41, 3018-3024

Simple and Easy Method to Evaluate Uptake Potential of Nanoparticles in Mammalian Cells Using a Flow Cytometric Light Scatter Analysis

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Many classes of nanoparticles have been synthesized and widely applied, however, there is a serious lack of information concerning their effects on human health and the environment. Considering that their use will increase, accurate and cost-effective measurement techniques for characterizing “nanotoxicity” are required. One major toxicological concern is that nanoparticles are easily taken up in the human body**. In this study, we developed a method of evaluating the uptake potential of nanosized particles using flow cytometric light scatter**. Suspended titanium dioxide (TiO2) particles (5, 23, or 5000 nm) were added to Chinese hamster ovary cells. Observation by confocal laser scanning microscopy showed that the TiO2 particles easily moved to the cytoplasm of the cultured mammalian cells, not to the nucleus. The intensity of the side-scattered light revealed that the particles were taken up in the cells dose-, time-, and size-dependently. In addition, surfacecoating of TiO2 particles changed the uptake into the cells, which was accurately reflected in the intensity of the side-scattered light. The uptake of other nanoparticles such as silver (Ag) and iron

oxide (Fe3O4) also could be detected. This method could be used for the initial screening of the uptake potential of nanoparticles as an index of "nanotoxicity".

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Mutation Research 628 (2007) 99–106

Cyto- and genotoxicity of ultrafine TiO2 particles in cultured human lymphoblastoid cells

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Titanium dioxide is frequently used in the production of paints, paper, plastics, welding rod-coating material, and cosmetics, because of its low toxicity. However, recent studies have shown that nano-sized or ultrafine TiO2 (UF-TiO2) (<100 nm in diameter) can generate pulmonary fibrosis and lung tumor in rats. Cytotoxicity induced by UF-TiO2 in rat lung alveolar macrophages was also observed. This generates great concern about the possible adverse effects of UF-TiO2 for humans. The cytotoxicity and genotoxicity of UF-TiO2 were investigated using the methyl tetrazolium cytotoxicity (MTT) assay, **the population growth assay, the apoptosis assay by flow cytometry**, the cytokinesis block micronucleus (CBMN) assay, the comet assay, and the hypoxanthineguanine phosphoribosyltransferase (*HPRT*) gene mutation assay. WIL2-NS cells were incubated for 6, 24 and 48 h with 0, 26, 65 and 130 _g/ml UF-TiO2. Significant decreases in viability were seen in the MTT assay at higher doses; for example, 61, 7 and 2% relative viability at 130 _g/ml for 6, 24 and 48-h exposure ($P < 0.01$). A dose-dependent relationship was observed, while a time-dependent relationship was seen only at the highest dose (130 _g/ml) after exposure for 24 and 48 h. Treatment with 130 _g/ml UF-TiO2 induced approximately 2.5-fold increases in the frequency of micronucleated binucleated cells ($P < 0.01$). In addition, a significant reduction in the cytokinesis block proliferation index was observed by the CBMN assay ($P < 0.05$). In the comet assay, treatment with 65 _g/ml UF-TiO2 induced approximately 5-fold increases in olive tail moment ($P < 0.05$). In the HPRT mutation assay, treatment with 130 _g/ml UF-TiO2 induced approximately 2.5-fold increases in the mutation frequency ($P < 0.05$). The results of this study indicate that UF-TiO2 can cause genotoxicity and cytotoxicity in cultured human cells.

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Inhalation Toxicology June 2010, Vol. 22, No. 7, Pages 593-600 , DOI 10.3109/08958371003596587

Low-level ozone exposure induces airways inflammation and modifies cell surface phenotypes in healthy humans

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The effects of low-level ozone exposure (0.08 ppm) on pulmonary function in healthy young adults are well known; however, much less is known about the inflammatory and immunomodulatory effects of low-level ozone in the airways. **Techniques such as induced sputum and flow cytometry make it possible to examine airways inflammatory responses and changes in immune cell surface phenotypes following low-level ozone exposure.** The purpose of this study was to determine if exposure to 0.08 parts per million ozone for 6.6 h induces inflammation and modifies immune cell surface phenotypes in the airways of healthy adult subjects. Fifteen normal volunteers underwent an established 0.08 part per million ozone exposure protocol to characterize the effect of ozone on airways inflammation and immune cell surface phenotypes. Induced sputum and flow cytometry were used to assess these endpoints 24 h before and 18 h after exposure. The results showed that exposure to 0.08 ppm ozone for 6.6 h induced increased airway neutrophils, monocytes, and dendritic cells and modified the expression of CD14, HLA-DR, CD80, and CD86 on monocytes 18 h following exposure. Exposure to 0.08 parts per million ozone is associated with increased airways inflammation and promotion of antigen-presenting cell phenotypes 18 hours following exposure. These findings need to be replicated in a similar experiment that includes a control air exposure.

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Toxicology Letters 172 (2007) 48–59

Cytokines and other immunological biomarkers in children's environmental health studies

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Environmental exposures (e.g. pesticides, air pollution, and environmental tobacco smoke) during prenatal and early postnatal development have been linked to a growing number of childhood diseases including allergic disorders and leukemia. Because the immune response plays a critical role in each of these diseases, it is important to study the effects of toxicants on the developing immune system. Children's unique susceptibility to environmental toxicants has become an important focus of the field of immunotoxicology and the use of immune biomarkers in molecular epidemiology of children's environmental health is a rapidly expanding field of research. In this review, we discuss how markers of immune status and immunotoxicity are being applied to pediatric studies, with a specific focus on the **various methods used to analyze T-helper-1/2 (Th1/Th2) cytokine profiles**. Furthermore, we review recent data on the effects of children's environmental exposures to volatile organic compounds, metals, and pesticides on Th1/Th2 cytokine profiles and the associations of Th1/Th2 profiles with adverse health outcomes such as pediatric respiratory diseases, allergies, cancer and diabetes. Although cytokine profiles are increasingly used in children's studies, there is still a need to acquire distribution data for different ages and ethnic groups of healthy children. These data will contribute to the validation and standardization of cytokine biomarkers for future studies. Application of immunological markers in epidemiological studies will improve the understanding of mechanisms that underlie associations between environmental exposures and immune-mediated disorders.

BH Note: **TH1/TH2 ratios and cytokine profile can be determined using flow cytometry.**

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Scandinavian Journal of Clinical and Laboratory Investigation
Vol. 69, No. 5, September 2009, 537–544

Patients referred to an indoor air health clinic: exposure to water-damaged buildings causes an increase of lymphocytes in bronchoalveolar lavage and a decrease of CD19 leucocytes in peripheral blood

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Background: Respiratory and other symptoms are often associated with exposure to microbes present in water-damaged buildings. ***Material and methods:*** We examined 82 consecutive patients referred to the Indoor Air Clinic, Helsinki University Hospital, due to symptoms suspected of having been caused by long-term exposure to water damage in the home or workplace. Exposure to water damage was assessed by building inspections and microbial analyses as needed. Bronchoalveolar lavage, lung function measurements, skin prick tests to inhalant allergens and radiological examinations were performed in all patients. Leucocyte subsets in peripheral blood were analysed in 35 patients. ***Results:*** Marked water damage was detected in the homes or workplaces of 47 (59%) patients; the remaining 34 patients formed the control group. The exposed group expressed more symptoms in total than the control group: fatigue, conjunctival symptoms, rhinitis with sinusitis, recurrent bronchitis and asthma were more common in the exposed group, but a signifi cant difference was seen only for headache. In BAL (bronchoalveolar lavage) samples, lymphocytes represented 25% of the total cell population in non-smoking-exposed patients compared with 12% in control patients (p_ 0.004). In peripheral blood, CD19 leucocytes were signifi cantly decreased in the exposed group (7.5% versus 12.3%; p _ 0.01). ***Conclusions:*** Confirmed exposure to water damage was associated with an increase in symptoms. **Exposure to water damage caused a significant change in the cellular composition in BAL fluid (lymphocytosis) and blood (decrease of CD19 cells).** The depletion of CD19 leucocytesin peripheral blood may indicate an active immune response in the lungs.

BH Note: Lymphocyte characterization determined using flow cytometry

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The American Journal of Gastroenterology Volume 104, January 2009 p. 102 - 109

Expression of the High-Affinity IgG Receptor FcRI (CD64) in Patients With Inflammatory Bowel Disease: A New Biomarker for Gastroenterologic Diagnostics

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We sought to determine the quantitative expression of the high-affi nity Fc receptor (CD64) on polymorphonuclear neutrophils (PMNs) in infl ammatory and functional conditions of the intestine and

investigated its correlation with clinical and biological parameters of inflammation. **The quantitative expression of CD64 was determined by fluorescence-activated cell sorting analysis in patients with active or inactive inflammatory bowel disease (IBD, *n* = 76), infectious enterocolitis, lactose and / or fructose intolerance, and healthy subjects.** The quantitative expression of CD64 in patients with active IBD (3,398 ± 3,589 molecules per PMN,(*n* = 27) was signifi cantly higher than in healthy subjects (607 ± 265, *n* = 28, *P* < 0.001) or in patients with lactose / fructose intolerance (531 ± 150, *n* = 32, *P* < 0.001). The expression of CD64 correlated signifi cantly with C-reactive protein (CRP, 0.65, *P* < 0.0001), Crohn ' s disease activity index (CDAI, 0.53, *P* < 0.0001), and colitis activity index (CAI, 0.63, *P* < 0.0001) in patients with IBD. With a cutoff point of 800, CD64 had a sensitivity of 88 % and a specifi city of 93 % in discriminating between lactose / fructose intolerance and active IBD. The quantitative expression of CD64 in patients with infectious enterocolitis (19,190 ± 8,920, *n* = 22) was signifi cantly higher than in patients with active IBD (*P* < 0.001). With a cutoff point of 10,000, CD64 showed a sensitivity of 96 % and a specifi city of 97 % in discriminating between infectious enterocolitis and active IBD. CD64 could serve as a valuable tool to discriminate between IBD, infectious enterocolitis, and functional intestinal disorders.

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Nature Protocols Vol. 2, No. 9, 2007 p. 2295-2301

Simultaneous detection of apoptosis and mitochondrial superoxide production in live cells by flow cytometry and confcal microscopy

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Annexin V and Sytox Green are widely used markers to evaluate apoptosis in various cell types using flow cytometry and fluorescent microscopy. **Recently, a novel fluoroprobe MitoSOX Red was introduced for selective detection of superoxide in the mitochondria of live cells and was validated for confocal microscopy and flow cytometry.** This protocol describes simultaneous measurements of mitochondrial superoxide generation with apoptotic markers (Annexin V and Sytox Green) by both flow cytometry and confocal microscopy in endothelial cell lines. The advantages of the described flow cytometry method over other cell-based techniques are the tremendous speed (1–2 h), exquisite precision and the possibility of simultaneous quantitative measurements of mitochondrial superoxide generation and apoptotic (and other) markers, with maximal preservation of cellular functions. This method combined with fluorescent microscopy may be very useful to reveal important spatial–temporal changes in mitochondrial superoxide production and execution of programmed cell death in virtually any cell type.

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Methods in Molecular Biology: Methods and Protocols 2010:594:57-72

Advanced Protocols in Oxidative Stress II

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Cells constantly generate reactive oxygen species (ROS) during aerobic metabolism. The ROS generation plays an important protective and functional role in the immune system. The cell is armed with a powerful

antioxidant defense system to combat excessive production of ROS. Oxidative stress occurs in cells when the generation of ROS overwhelms the cells' natural antioxidant defenses. ROS and the oxidative damage are thought to play an important role in many human diseases including cancer, atherosclerosis, other neurodegenerative diseases and diabetes. Thus, establishing their precise role requires the ability to measure ROS accurately and the oxidative damage that they cause. There are many methods for measuring free radical production in cells. **The most straightforward techniques use cell permeable fluorescent and chemiluminescent probes. 2′-7′-Dichlorodihydrofluorescein diacetate (DCFH-DA) is one of the most widely used techniques for directly measuring the redox state of a cell.** It has several advantages over other techniques developed. It is very easy to use, extremely sensitive to changes in the redox state of a cell, inexpensive and can be used to follow changes in ROS over time.

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Environmental Pollution 156 (2008) 732–738

Effects of methylmercury exposure on glutathione metabolism, oxidative stress, and chromosomal damage in captive-reared common loon (Gavia immer) chicks

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We quantified the level of dietary mercury (Hg), delivered as methylmercury chloride (CH3HgCl), associated with negative effects on organ and plasma biochemistries related to glutathione (GSH) metabolism and **oxidative stress, and chromosomal damage** in captive-reared common loon (Gavia immer)chicks reared from hatch to 105 days. Mercury-associated effects related to oxidative stress and altered glutathione metabolism occurred at 1.2 mg Hg/g and 0.4 mg Hg/g, an ecologically relevant dietary mercury level, but not at 0.08 mg Hg/g. Among the variables that contributed most to dissimilarities in tissue chemistries between control and treatment groups were increased levels of oxidized glutathione (GSSG), GSH peroxidase, and the ratio of GSSG to GSH in brain tissue; increased levels of hepatic GSH; and decreased levels of hepatic glucose-6-phosphate dehydrogenase (G-6-PDH). Our results also suggest that chronic exposure to environmentally relevant dietary Hg levels did not result in statistically significant somatic chromosomal damage in common loon chicks.

BH Note: Genotoxicity determined using flow cytometric methods.

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Environmental Toxicology and Chemistry**,** Vol. 16, No. 3, pp. 576–581, 1997

Immunotoxicity of environmentally relevant mixtures of polychlorinated aromatic hydrocarbons with methyl mercury on rat lymphocytes in vitro

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The immunosuppressive effects of methyl mercury (MHg), polychlorinated biphenyls (PCBs), polychlorinated dibenzo*p*-dioxins (PCDDs), and dibenzofurans (PCDFs) are well established at high exposure levels but unclear at low exposure levels. We exposed Fischer 344 rat splenocytes, thymocytes, and peripheral blood lymphocytes in vitro for 72 h to MHg (0.1, 2 mg/ml), PCDD/PCDF mixtures (1, 15 pg/ml) of three PCDDs (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin,and 1,2,3,4,7,8-hexachlorodibenzo-*p*-dioxin) and two PCDFs (2,3,7,8-tetrachlorodibenzofuran and 1,2,3,7,8-pentachlorodibenzofuran),three Aroclort (1242, 1254, 1260), PCB mixtures (0.01, 0.5 mg/ml), or combinations of MHg/PCB/PCDD/PCDF mixtures Mitogenic responses of lymphocytes to concanavalin A, phytohemagglutinin, or lipopolysaccharide/dextran sulfate were determined by 3H-thymidine uptake; **cytotoxicity and intracellular Ca21 were determined by flow cytometry**. Methylmercury (2 mg/ml and PCB/PCDD/PCDF mixtures with 2 mg/ml MHg decreased the viability of splenocytes to 57 and 40% at 4 and 24 h, respectively. Basal intracellular calcium ion levels were unaffected by the treatments. Methylmercury suppressed the responses of lymphocytes to T and B cell mitogens. All combinations of MHg/PCB/PCDD/PCDF mixtures decreased mitogenic responses to levels similar to those to MHg alone. In contrast, PCB and PCDD/PCDF mixtures did not suppress but augmented responses of splenocytes and peripheral blood lymphocytes to T cell mitogens. Overall, no interactive toxicity was observed with MHg/PCB/PCDD/PCDF mixtures on cytotoxicity and lymphocyte mitogenic responses. Therefore, MHg may pose a greater threat than organochlorines to the mammalian immune system.

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Toxicology in Vitro 24 (2010) 1119–1125

Comparison of immunotoxic effects induced by the extracts from methanol and gasoline engine exhausts in vitro

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Gasoline engine exhaust has been considered as a major source of air pollution in China. Due to lower cyto- and geno-toxicity effects of methanol engine exhaust, methanol is regarded as a potential substitute for gasoline. We have previously compared cyto- and geno-toxicities of gasoline engine exhaust with that of methanol engine exhaust in A549 cells (Zhang et al., 2007).To characterize the immunotoxic effects for gasoline and methanol engine exhausts in immune cell, in this study, we further compared effects of gasoline and methanol engine exhausts on immune function in RAW264.7 cell and rabbit alveolar macrophages. **Results showed that both gasoline and methanol engine exhaust could evidently inhibit RAW264.7 cell proliferation, promote RAW264.7 cell apoptosis,** decrease E-rosette formation rate and inhibit anti-tumor effects of alveolar macrophages, at the same time, these effects of gasoline engine exhaust were far stronger than those of methanol engine exhaust. In addition, gasoline engine exhaust could significantly inhibit activities of ADCC of alveolar macrophages, but methanol engine exhaust could not. These results suggested that both gasoline and methanol engine exhausts might be immunotoxic atmospheric pollutants, but some effects of gasoline engine exhaust on immunotoxicities may be far stronger than that of methanol engine exhaust.

BH Note: **Cell proliferation and apoptosis determined by flow cytometry.**

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Journal of Environmental Sciences 2010, 22(2) 297–303

Reproductive toxicity of organic extracts from petrochemical plant effluents discharged to the Yangtze River, China

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Water pollution of the Yangtze River in China became one of challenges that the government is facing today. Increasing numbers of petrochemical plants were built along the river in past decades, and numbers of organic chemicals were discharged into the river. Our goal was to establish *in vitro* system on rat sertoli cells, spermatogenic cells and leydig cells to investigate the reproductive toxicity potential induced by organic extracts from petrochemical effluents. Our results showed that the organic extract depressed the viability ($p < 0.01$) and destroyed the plasma membrane integrity of sertoli cells and spermatogenic cells to a certain degree. Accordingly, proportion of early apoptotic sertoli cells and late apoptotic spermatogenic cells increased significantly. Although significant morphological changes were not detected for leydig cells, the extract was observed to inhibit their testosterone production ($p< 0.01$). Sertoli cells and spermatogenic cells appeared to be more sensitive and maybe the main targets of the key toxins. These results suggested that the *in vitro* system on rat testicular cells may be useful to predicate reproductive toxicity potential of organic extracts from petrochemical effluents. More attention should be paid to the petrochemical effluents, because long-term accumulation of these organic compounds in organisms may cause spermatogenesis malfunction and testosterone reduction.

BH Note: **Apoptotic and necrotic cells determined by flow cytometry.**

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Environ Toxicol 25: 188–197, 2010.

Polycyclic Aromatic Hydrocarbons from Diesel Emissions Exert Proallergic Effects in Birch Pollen Allergic Individuals Through Enhanced Mediator Release from Basophils

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Background: Diesel exhaust particles (DEPs) act as adjuvants in the immune system and

contribute to the increased prevalence and morbidity of asthma and allergic rhinitis. Polycyclic aromatic hydrocarbons (PAHs) are major components of DEPs, which may be involved in the induction and enhancement of proallergic processes. In this study we explored adjuvant effects of DEP-PAHs on activation parameters of human basophils, fostering allergic inflammation through the release of preformed or granule-derived mediators. Methods: Heparinized blood samples from birch pollen allergic and control donors were stimulated with Bet v 1, the major allergen of birch pollen grains, alone or together with a mixture of 16 environmental prominent PAHs (EPA-PAH standard). **Flow cytometric analysis was performed for quantitative determination of PAH-enhanced basophil activation**. To assess direct PAH effects on basophils, enriched cultures from both donor groups were exposed to benzo[a]pyrene (B[a]P) or phenanthrene(Phe), two major DEP-PAHs, with and without allergen. Supernatants were assayed for IL-4 and IL-8 secretion and histamine release by means of ELISA. Results: At environmental relevant exposure levels EPA-PAH standard synergized with antigen and significantly enhanced basophil activation of all birch pollen allergic individuals up to 95%. Single PAHs significantly drove IL-8 secretion from sensitized basophils of all patients tested, and there was no further enhancement by addition of rBet v 1. B[a]P and Phe also significantly induced IL-4 secretion, a key factor for Th2 development, from purified sensitized basophils in the absence of antigen suggesting an adjuvant role of DEP-PAHs in allergic sensitization. None of the basophil samples from healthy controls showed any PAH effect on mediator release. Conclusion: DEPPAHs exert proallergic effects on sensitized basophils in an allergen independent fashion, suggesting a potential role of these pollutants for the allergic breakthrough in atopic individuals, who have not developed an allergic disease yet.

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Mutation Research 630 (2007) 78–91

In vitro micronucleus assay scored by flow cytometry provides a comprehensive evaluation of cytogenetic damage and cytotoxicity

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This laboratory has previously reported on the development of a flow cytometry-based method for scoring *in vitro* micronuclei in mouse lymphoma (L5178Y) cells [S.L. Avlasevich, S.M. Bryce, S.E. Cairns, S.D. Dertinger, *In vitro* micronucleus scoring by flow cytometry: differential staining of micronuclei *versus* apoptotic and necrotic chromatin enhances assay reliability, Environ. Molec. Mutagen. 47 (2006) 56–66]. With this method, necrotic and mid/late stage apoptotic cells are labeled with the fluorescent dye ethidium monoazide. Cells are then washed, stripped of their cytoplasmic membranes, and incubated with RNase plus a pan-nucleic acid dye (SYTOX Green). This process provides a suspension of free nuclei and micronuclei that are differentially stained relative to chromatin associated with dead/dying cells. The current report extends this line of investigation to include the human cell line TK6. Additionally, methods are described that facilitate simultaneous quantitative analysis of cytotoxicity, perturbations to the cell cycle, and what we hypothesize is aneuploidization. This comprehensive cytogenetic damage assay was evaluated with the following diverse agents: etoposide, ionizing radiation, methyl methanesulfonate, vinblastine, ethanol, and staurosporine. Cells were harvested after 30 h of continuous treatment (in the case of chemicals), or following graded doses of radiation up to 1Gy. Key findings include the following: (1) Significant discrepancies in top dose selection were found for five of the six agents studied when relative survival measurements were based on Coulter counting *versus* flow cytometry. (2) Both microscopy- and flow cytometry-based scoring methods detected dose-dependent micronucleus formation for the four genotoxic agents studied, whereas no significant increases were observed for the presumed non-genotoxicants ethanol and staurosporine when top dose selection was based on flow cytometric indices of cytotoxicity. (3) SYTOX and ethidium monoazide fluorescence signals conveyed cell cycle and cell death information,

respectively, and appear to represent valuable aids for interpreting micronucleus data. (4) The frequency of hypodiploid nuclei increased in response to each of the genotoxic agents studied, but not following exposure to ethanol or staurosporine. **Collectively, these results indicate that a comprehensive assessment of genotoxicity and other test article-induced toxicities can be acquired simultaneously using a simple two-color flow cytometry-based technique**.

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Toxicology in Vitro 21 (2007) 176–182

Cytomics: A multiparametric, dynamic approach to cell research

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Cytomics aims to determine the molecular phenotype of single cells. Within the context of the -omics, cytomics allows the investigation of multiple biochemical features of the heterogeneous cellular systems known as the cytomes. Cytomics can be considered as the science of single cell-based analyses that links genomics and proteomics with the dynamics of cell and tissue function, as modulated by external influences. Inherent to cytomics are the use of sensitive, scarcely invasive, fluorescence-based multiparametric methods and the event-integrating concept of individual cells to understand the complexity and behaviour of tissues and organisms. Among cytomic technologies, flow cytometry, confocal laser scanning microscopy and laser capture microdissection are of great relevance. Other recent technologies based on single cell bioimaging and bioinformatic tools become important in drug discovery and toxicity testing, because of both high content and high-throughput. The multiparametric capacity of cytomics is very useful for the identification, characterization and isolation of stem cell populations**. In our experience, flow cytometry is a powerful and versatile tool that allows quantitative analysis of single molecules, prokaryotic and eukaryotic cells for basic, biotechnological, environmental and clinical studies.** The dynamic nature of cytomic assays leads to a real-time kinetic approach based on sequential examination of diVerent single cells from a population undergoing a dynamic process, the in fluxo level. Finally, cytomic technologies may provide in vitro methods alternative to laboratory animals for toxicity assessment.

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Mutation Research 649 (2008) 101–113

Comparison of flow cytometry- and microscopy-based methods for measuring micronucleated reticulocyte frequencies in rodents treated with nongenotoxic and genotoxic chemicals

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The development of automated flow cytometric (FCM) methods for evaluating micronucleus (MN) frequencies in erythrocytes has great potential for improving the sensitivity, reproducibility, and throughput of the traditional *in vivo* rodent MN assay that uses microscopy-based methods for data collection. Although some validation studies of the FCM evaluation methods have been performed, a comprehensive comparison of these two data collection methods under routine testing conditions with a variety of compounds in multiple species has not been conducted. Therefore, to determine if FCM evaluation of MN frequencies in rodents was an acceptable alternative to traditional manual scoring methods in our laboratory, we conducted a comparative evaluation of MN-reticulocyte (MN-RET) frequencies determined by FCM- and microscopy-based scoring of peripheral blood and bone marrow samples from B6C3F1 mice and Fisher 344 rats. Four known inducers of MN (cyclophosphamide, ethyl methanesulfonate, vincristine sulfate, acrylamide) were assayed in bone marrow and peripheral blood of both mice and rats. In addition, MN-RET frequencies were measured in bone marrow (microscopy) and peripheral blood (FCM) of mice treated with five nongenotoxic chemicals (*S*-adenosylmethionine chloride, cefuroxime, diphenolic acid, 3-amino-6-methylphenol, pentabromodiphenyl oxide). No significant differences were observed between results obtained by the two methods in either species. **These results support the use of FCM for determining MN-RET frequency in rodents after chemical exposure.**

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Water Research (2010)1-9

Measurement and interpretation of microbial adenosine tri-phosphate (ATP) in aquatic environments

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There is a widespread need for cultivation-free methods to quantify viability of natural microbial communities in aquatic environments. Adenosinetri-phosphate(ATP) is the energy currency ofall living cells,and therefore a useful indicator of viability. A luminescence based ATPkit/protocol was optimised in order to detect ATP concentrations as low as 0.0001nM with a standard deviation of <5%. Using this method, more than 100 water samples from a variety of aquatic environments (drinking water,groundwater, bottled water,river water,lake water and waste water effluent) were analysed for extra- cellular ATPand microbial ATP in comparison with flow-cytometric (FCM) parameters. Microbial ATP concentrations ranged between 3%and 97% of total ATP concentrations, and correlated well (R2 = 0.8) with the concentrations of intact microbial cells (afterstaining with propidiumiodide).From this correlation,we calculated an average ATP-per-cellvalue of 1.75 x 10^{-10} nmol/cell. **An even better correlation(R2 = 0.88) was observed between intact biovolume (derived from FCM scatter data) and microbial ATPconcentrations, and an average ATP-per-biovolume value of 2.95 x 10^{-9} nmol/mm^3 was calculated.**These results support the use of ATP analysis for both routine monitoring and research purposes, and contribute towards a better interpretation of ATP data.

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Environmental Pollution 157 (2009) 3042–3050

Riboflavin content of coelomocytes in earthworm (Dendrodrilus rubidus) field populations as a molecular biomarker of soil metal pollution

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The effect of Pb + Zn on coelomocyte riboflavin content in the epigeic earthworm Dendrodrilus rubidus inhabiting three metalliferous soils and one reference soil was measured by flow cytometry and spectrofluorimetry. A reciprocal polluted/unpolluted worm transfer experiment (4-week exposure) was also performed. High proportions of autofluorescent eleocytes were counted in worms from all localities, but intense riboflavin-derived autofluorescence was detectable only in reference worm eleocytes. Other findings were: (i) fluorophore(s) other than riboflavin is/are responsible for eleocyte autofluorescence in residents of metalliferous soils; (ii) riboflavin content was reduced in the eleocytes of worms transferred from unpolluted to metal-polluted soil; (iii) the riboflavin content of D. rubidus eleocytes is a promising biomarker of exposure; (iv) COII mitochondrial genotyping revealed that the reference population is genetically distinct from the three mine populations; (v) metal exposure rather than genotype is probably the main determinant of inter-population differences in eleocyte riboflavin status.

BH Note: Coelomocytes are a measure of innatue immunity in earthworms. Riboflavin is an antioxidant capable of stimulating the immune response in humans and animals.

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Optimization of FDA–PI method using flow cytometry to measure metabolic activity of the cyanobacteria, Microcystis aeruginosa

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A rapid toxicity test based on inhibition of esterase activity in the harmful freshwater microalgae – Microcystis aeruginosa was developed using flow cytometry. The hydrolysis rate of fluorescein diacetate (FDA) by intracellular esterase to fluorescein was used to indicate the metabolic activity of algae. Uptake of FDA was optimized at different concentrations and incubation times. Propidium iodide (PI) was utilized to assess cell membrane integrity. The optimized FDA/PI staining dosages were 10 mg/L and 10 lM, respectively, lower than the reported concentrations. Correspondingly, the proper incubation time was 14–21 min at the optimal FDA dosage determined in this study. A new procedure based on optimized FDA/PI condition, called ‘‘whole algal culture flow cytometry with fluorescence triggering”, was developed for short-term bioassays. This new procedure, taking account of working conditions such as pH and impure cultures, is able to avoid algal cell damages in sample preparation and separate algal cells from non-algal particles by fluorescence triggering. This newly-developed procedure was then used to assess the toxicity of copper on M. aeruginosa in a short-term exposure (36 h). As copper concentrations increased, it was found that the esterase activity decreased in a concentration-dependent manner with increased membrane fragments. Moreover, esterase activity was a good indicator of copper toxicity in M. aeruginosa. The EC50 value based on mean fluorescence intensity (MFI) was 123.3 lg/L (95% confidence limits 101.5–146.2 lg/L). Therefore, the new-developed procedure could be used for sublethal endpoints

detection, and has the potential to be a rapid and cost-effective bioassay for selecting M. aeruginosa control methods or exploring the M. aeruginosa activity inhibition mechanism.

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Water Research 44 (2010) 3807-3818

Direct quantification of bacterial biomass in influent, effluent and activated sludge of wastewater treatment plants by using flow cytometry

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A rapid multi-step procedure, potentially amenable to automation, was proposed for quantifying viable and active bacterial cells, estimating their biovolume using flow cytometry (FCM) and to calculate their biomass within the main stages of a wastewater treatment plant: raw wastewater, settled wastewater, activated sludge and effluent. Fluorescent staining of bacteria using SYBR-Green I þ Propidium Iodide (to discriminate cell integrity or permeabilisation) and BCECF-AM (to identify enzymatic activity) was applied to count bacterial cells by FCM. A recently developed specific procedure was applied to convert Forward Angle Light Scatter measured by FCM into the corresponding bacterial biovolume. This conversion permits the calculation of the viable and activebacterial biomass in wastewater, activated sludge and effluent, expressed as Volatile Suspended Solids (VSS) or particulate Chemical Oxygen Demand (COD). Viable bacterial biomass represented only a small part of particulate COD in raw wastewater (4.8 _ 2.4%), settled wastewater (10.7 _ 3.1%), activated sludge (11.1 _ 2.1%) and effluent (3.2 _ 2.2%). Active bacterial biomass counted for a percentage of 30e47% of the viable bacterial biomass within the stages of the wastewater treatment plant.