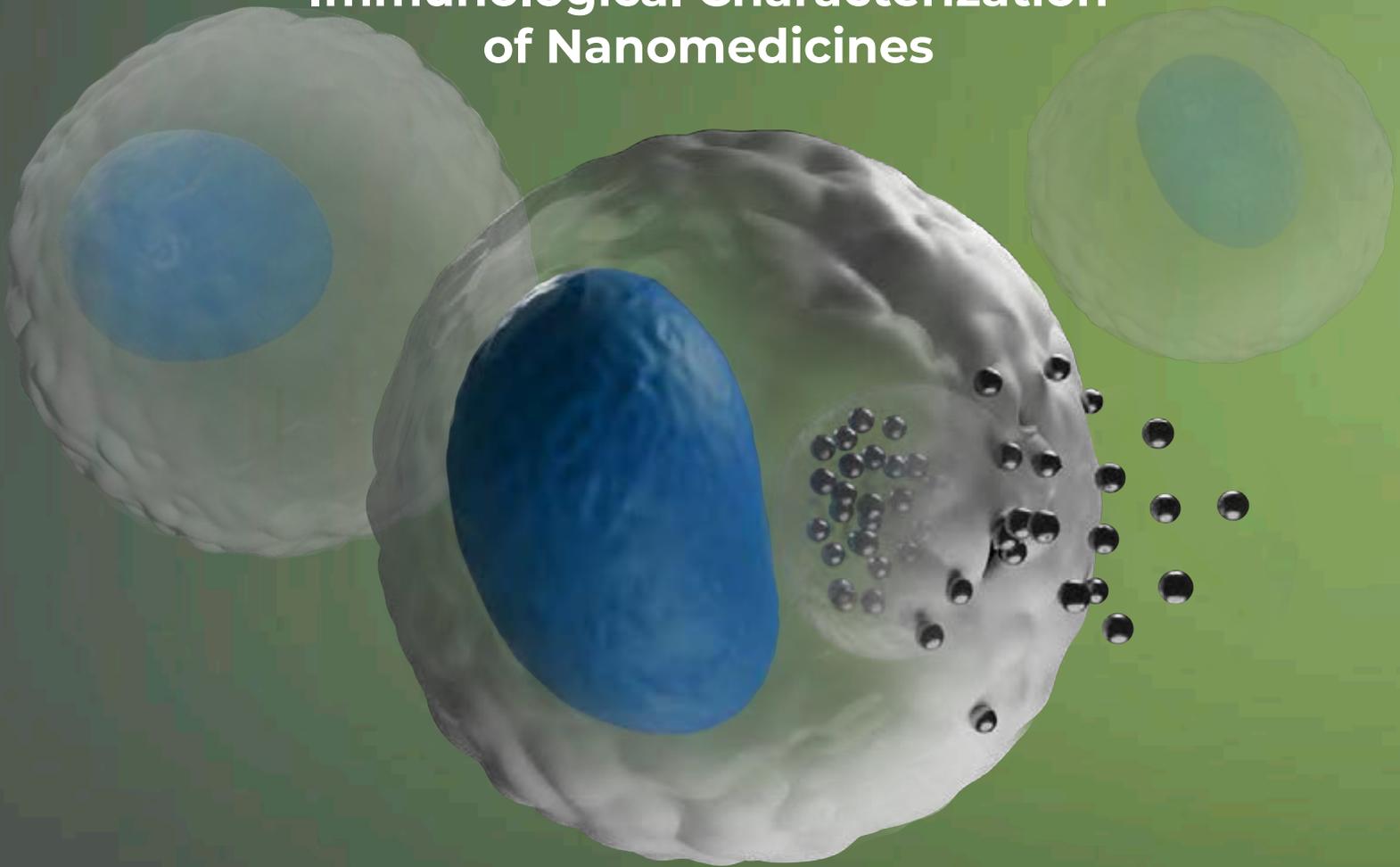


Immunological Characterization of Nanomedicines



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IMMUNOLOGICAL CHARACTERIZATION OF NANOMEDICINES AT THE NCL

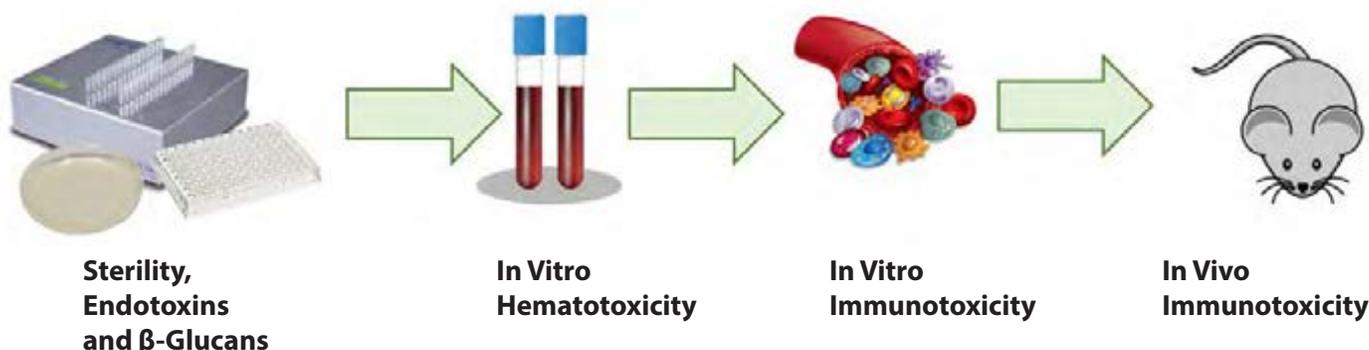
Nanoformulated drugs often induce a variety of toxicities and reactions originating from nanoparticle interaction with various components of the immune system. Contamination with endotoxin and bacteria, overt cytokine release, complement activation, leukocyte responses and perturbation of blood coagulation pathways are among the most frequent reasons halting the preclinical development of nanomedicines. Therefore, screening for these toxicities early in the development process can not only save the developer resources but can also prevent adverse reactions in patients when the formulation reaches clinical trials.

Immunological characterization of nanomedicines as part of the NCL's Assay Cascade characterization service aims to do just this. It spans both in vitro and in vivo characterization, as well as analysis of not only the nanoformulation, but in certain cases, precursor formulations, control formulations, individual components and formulation constituents.

NCL immunological characterization begins with assessment of sterility, endotoxin and beta-glucan levels, then moves to analysis of in vitro hematological compatibility and immunotoxicity, and finally evaluation of in vivo immunotoxicity. Each of these are described in more detail on the following pages.

The NCL has more than 40 protocols available for the immunological evaluation of nanomaterials. Importantly, the assays selected for your formulation will be chosen based on current knowledge about the nanopatform, the active pharmaceutical ingredient, and the critical gaps in your developmental path. Having tested more than 400 different nanoparticles with a variety of platforms (metallic, liposomes, polymers, proteins, micelles, DNA and RNA nanostructures, carbon nanotubes, etc.) and active ingredients (small molecules, nucleic acids, peptides, proteins, etc.), the NCL can assist with identifying the most appropriate assays for your nanomedicine formulation.

NCL's Tiered Approach To Evaluating Nanoparticle Immunotoxicity



IMMUNE-MODULATING IMPURITIES

Immunological characterization of nanomaterials involves an in-depth examination of not only the direct immunological consequences of nanoparticle treatment, but also detection of potential innate immunity-modulating impurities in the sample. Every particle that comes into the NCL's Assay Cascade characterization testing program will be screened for microbial contamination, as well as for levels of endotoxin and beta-glucans.

Microbial Contamination

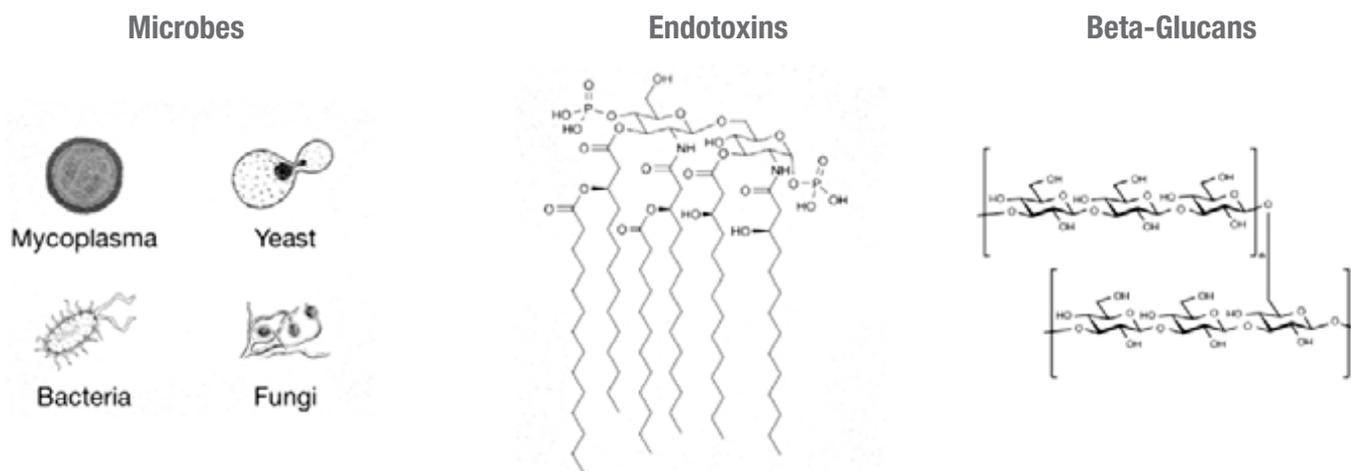
Bacteria and other microbes found in water, soil and as part of the human microflora are common contaminants in nanoformulations. Therefore, all formulations are screened for potential microbial contamination before being used in other tests, including screening on both agar plates and in culture media. In addition, any contaminants found are subjected to further analysis to identify the species, which may assist developers in identifying the source of contamination.

Endotoxins

Endotoxin, a natural component of gram-negative bacteria, is a common contaminant often found in starting materials and water supplies. High levels of endotoxin can confound interpretation of select in vitro immunotoxicity assays, and for certain products, effect the efficacy of the formulation. Therefore, NCL screens all samples for endotoxin levels using at least two Limulus Amoebocyte Lysate (LAL) assays, including appropriate inhibition-enhancement controls.

Beta-Glucans

Beta-glucan levels are of interest for biotechnology therapeutics and for complex formulations containing components with potential immunogenicity concerns. The level of beta-glucans in pharmaceuticals is not as tightly controlled as the levels of endotoxin due to the lower biological potency of beta-glucans. However, monitoring beta-glucan levels is informative for batch-to-batch consistency and is an emerging trend in the area of bio- and immunotherapeutics due to the contribution of beta-glucans to the immunogenicity issue.



Adapted from Neun et al, *Molecules*, **2020**, 25(15), 3367.

Relevant NCL Publications

Neun et al, *Molecules*, **2020**, 25(15), 3367. PMID: [32722261](https://pubmed.ncbi.nlm.nih.gov/32722261/)

Neun & Dobrovolskaia, *Journal of Visualized Experiments*, **2019**, 143, e58830. PMID: [30774143](https://pubmed.ncbi.nlm.nih.gov/30774143/)

Neun & Dobrovolskaia, *Methods in Molecular Biology*, Vol. 1628, **2018**, Humana Press, New York, NY, p. 23–33. PMID: [29039090](https://pubmed.ncbi.nlm.nih.gov/29039090/)

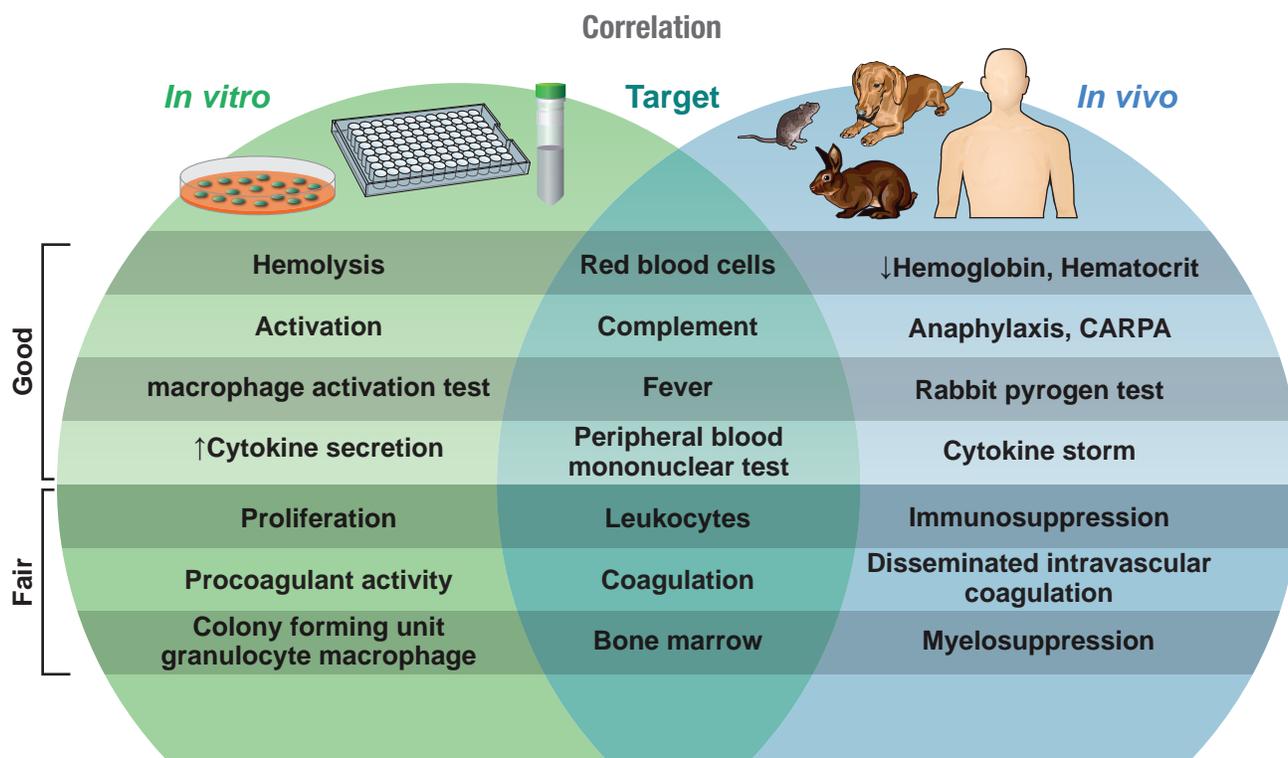
IN VITRO HEMATOLOGICAL COMPATIBILITY

Hematotoxicity of nanomaterials is tested in vitro using freshly drawn human blood from healthy donor volunteers. Tests routinely conducted at the NCL include in vitro analysis of nanoparticle effects on erythrocytes, platelets, the complement system and the plasma coagulation cascade. Nanoparticles are tested in a battery of assays assessing hemolysis, platelet aggregation, complement activation and plasma coagulation times at several concentrations using an estimated human dose, converted from the developers' previous animal studies, and include several concentrations below and above this estimated human dose. For more information on how in vitro test concentrations are selected, please see the description provided in Dobrovolskaia & McNeil, *J Control Release*, **2013**;172(2):456–66, and also discussed in individual NCL protocols, for example, NCL protocol ITA-1, https://ncl.cancer.gov/sites/default/files/NCL_Method_ITA-1.pdf.

While no in vitro test is entirely predictive of in vivo response for all test materials, available literature data and NCL experience reveal that many of these hematotoxicity/immunotoxicity tests have good in vitro-in vivo correlation and therefore can be used to evaluate the risk of nanomaterials inducing these toxicities in human patients.

Immunological Assays with In Vitro–In Vivo Correlation

In vitro assays with good/fair correlation to in vivo results can be used as a quicker and more effective approach to screening nanoformulations.



Adapted from Dobrovolskaia & McNeil, *J Control Release*, **2013**,172(2), 456–66.

Relevant NCL Publications

Dobrovolskaia et al, *Toxicol Appl Pharmacol*, **2016**, 299, 78–89. PMID: 26739622
 Dobrovolskaia & McNeil, *J Control Release*, **2013**, 172(2), 456–466. PMID: 23742883

IN VITRO IMMUNE CELL FUNCTION ASSAYS

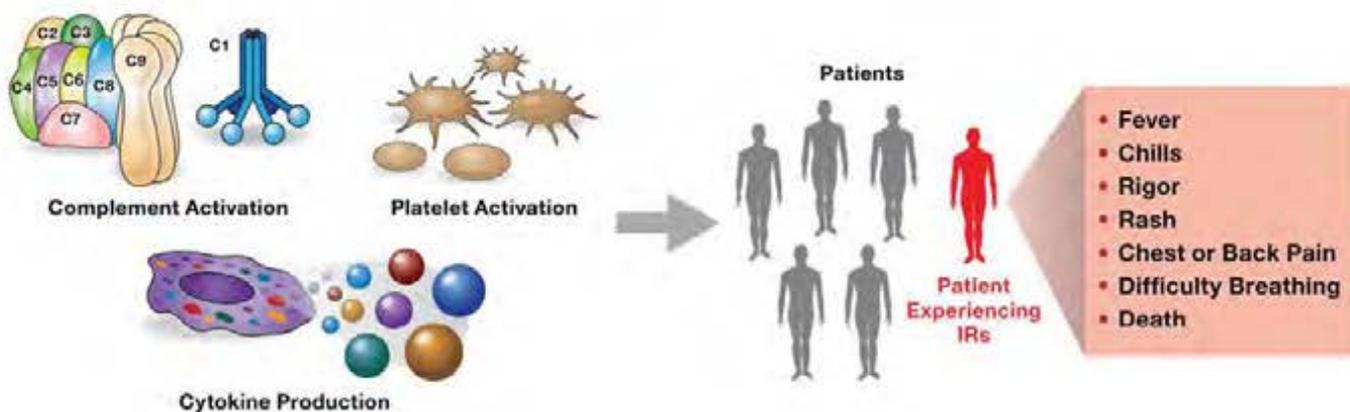
Immunotoxicity is one of the most common reasons for failure of nanomedicines in clinical trials; therefore, evaluation of potential adverse immune-mediated reactions is a critical component of the preclinical evaluation process. When conducted together, many of these assays can help investigators understand the potential risk, helping to mitigate potential safety concerns down the road.

The NCL has several in vitro methods established to assess the effect of nanoparticle treatment on immune cell function, including:

- Chemotaxis
- Phagocytosis
- Leukocyte Proliferation
- Leukocyte Procoagulant Activity
- Human Leukocyte Activation
- Cytokines, chemokines & interferons
- CFU-GM
- NO- Production
- Cytotoxicity of NK Cells
- Maturation of monocyte-derived Dendritic Cells

Common Immunotoxicities Resulting in Infusion Reactions

In vitro assays can be used to screen for these early in development.



The NCL also has several in vitro protocols to explore the mechanistic immunotoxicology of nanoformulations. These include:

- Detection of Intracellular Complement Activation in Human T Lymphocytes
- Detection of Nanoparticle-Mediated Total Oxidative Stress in T-Cells Using CM-H2DC-FDA Dye
- Detection of Mitochondrial Oxidative Stress in T-Cells Using MitoSOX Red Dye
- Detection of Changes in Mitochondrial Membrane Potential in T-Cells Using JC-1 Dye
- Detection of Antigen Presentation by Murine Bone Marrow-Derived Dendritic Cells
- Antigen-Specific Stimulation of CD8+ T-Cells by Murine Bone Marrow-Derived Dendritic Cells
- Detection of Naturally Occurring Antibodies to PEG and PEGylated Liposomes
- A panel of HEK-TLR reporter cell lines specific to the common innate-immunity modulating impurities triggering the activation of TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, or TLR9

IN VIVO IMMUNOTOXICITY

In addition to the in vitro assays for assessment of immunotoxicity, the NCL has several in vivo methods established to assess the immunotoxicity of nanoformulations in rodents.*



Adjuvanticity

With the increasing use of nanotechnology as vaccine adjuvants, this in vivo assessment can be used to evaluate and ultimately improve vaccine efficacy.

Autoimmunity and Autoinflammatory responses

Autoimmunity and autoinflammation are common side-effects of immunotherapies. As nanoparticles are being increasingly explored as immunotherapies, this study can be used to assess whether the nanotech carriers and formulations can break immunological tolerance and promote autoimmunity or induce autoinflammatory responses. These studies are conducted in models of chemically-induced systemic lupus erythematosus and psoriasis.

Colony forming unit granulocyte macrophage (CFU-GM)

Myelosuppression is a common toxicity of cytotoxic oncology drugs. CFU-GM is another test used at the NCL to assess nanoparticle immunosuppressive properties, and specifically to understand the toxicities to bone marrow pluripotent stem cells and their differentiation into granulocytes and macrophages.

Local lymph node assay (LLNA) / Local lymph node proliferation (LLNP)

These assays can be used to identify delayed-type hypersensitivity reactions in response to nanoparticle administration. The NCL tests formulations through repeat application on the skin of animals (LLNA) or by subcutaneous injection (LLNP) before collection of lymph nodes for analysis of activated lymphocytes.

Rabbit pyrogen test (RPT)

Pyrogenicity of nanomaterials can be assessed in vivo using the rabbit pyrogen test and is a useful assessment for nanomaterials interfering with the more common LAL test for endotoxins. The RPT is outsourced to an outside contract research organization.

T-cell dependent antibody responses (TDAR)

The TDAR assessment can be used to identify nanoformulations that exhibit immunosuppressive properties. While strong immunosuppression can be identified using standard in vitro and in vivo toxicological studies, moderate immunosuppression is best assessed using functional in vivo tests such as this.

*The Frederick National Laboratory for Cancer Research is accredited by AAALAC International and follows the Public Health Service Policy for the Care and Use of Laboratory Animals (Health Research Extension Act of 1985, Public Law 99-158, 1986). Animal care is provided in accordance with the procedures outlined in the Guide for Care and Use of Laboratory Animals (National Research Council, 1996; National Academy Press, Washington, D.C.). All animal protocols are approved by the FNLCR institutional Animal Care and Use Committee.

Relevant NCL Publications

Shah et al, *Prec Nanomed*, **2019**, 2(1), 249-255 [https://doi.org/10.33218/prnano2\(1\).181218.1](https://doi.org/10.33218/prnano2(1).181218.1)
Potter et al, *Methods Mol Biol*, **2018**, 1682, 161-172. PMID: 29039101
Neun & Dobrovolskaia, *Methods Mol Biol*, **2018**, 1682, 189-195. PMID: 29039103
Potter et al, *Methods Mol Biol*, **2018**, 1682, 197-210. PMID: 29039104
Zamboni et al, *Adv Drug Deliv Rev*, **2018**, 136-137, 82-96. PMID: 30273617

MEET THE EXPERT



Dr. Marina A. Dobrovolskaia, PhD, MBA, PMP

Director of Operations,
Head of Immunology

About Dr. Dobrovolskaia

Dr. Dobrovolskaia has been with the NCL since its founding and has been instrumental in bringing NCL to the forefront of immunological expertise as it pertains to nanomaterials. Marina has optimized more than 40 assays for the assessment of sterility, endotoxin, beta-glucans, hematotoxicity, and immunotoxicity of nanomaterials. She has more than 100 publications in the field and has collaborated with countless experts around the globe on structure-activity relationships and immunogenicity of nanomaterials.

Prior to joining the NCL, Marina worked as a Research Scientist at PPD Development, Inc., where she was responsible for the design, development and validation of bioanalytical ligand-binding assays to support pharmacokinetic and toxicity studies in a variety of drug development projects. She received her M.S. degree from the Kazan State University in Russia; Ph.D. from the N.N. Blokhin Cancer Research Center of the Russian Academy of Medical Sciences in Moscow, Russia; and MBA from Hood College in Frederick, MD. Since 2016, she is also a member of the Project Management Institute and a certified Project Management Professional.

Additional Resources

- Protocols for assays described here can be downloaded for free on the NCL's website: <https://ncl.cancer.gov/resources/assay-cascade-protocols>
- Additional publications on these topics are available here: <https://ncl.cancer.gov/resources/ncl-scientific-bibliography>

Contact Information

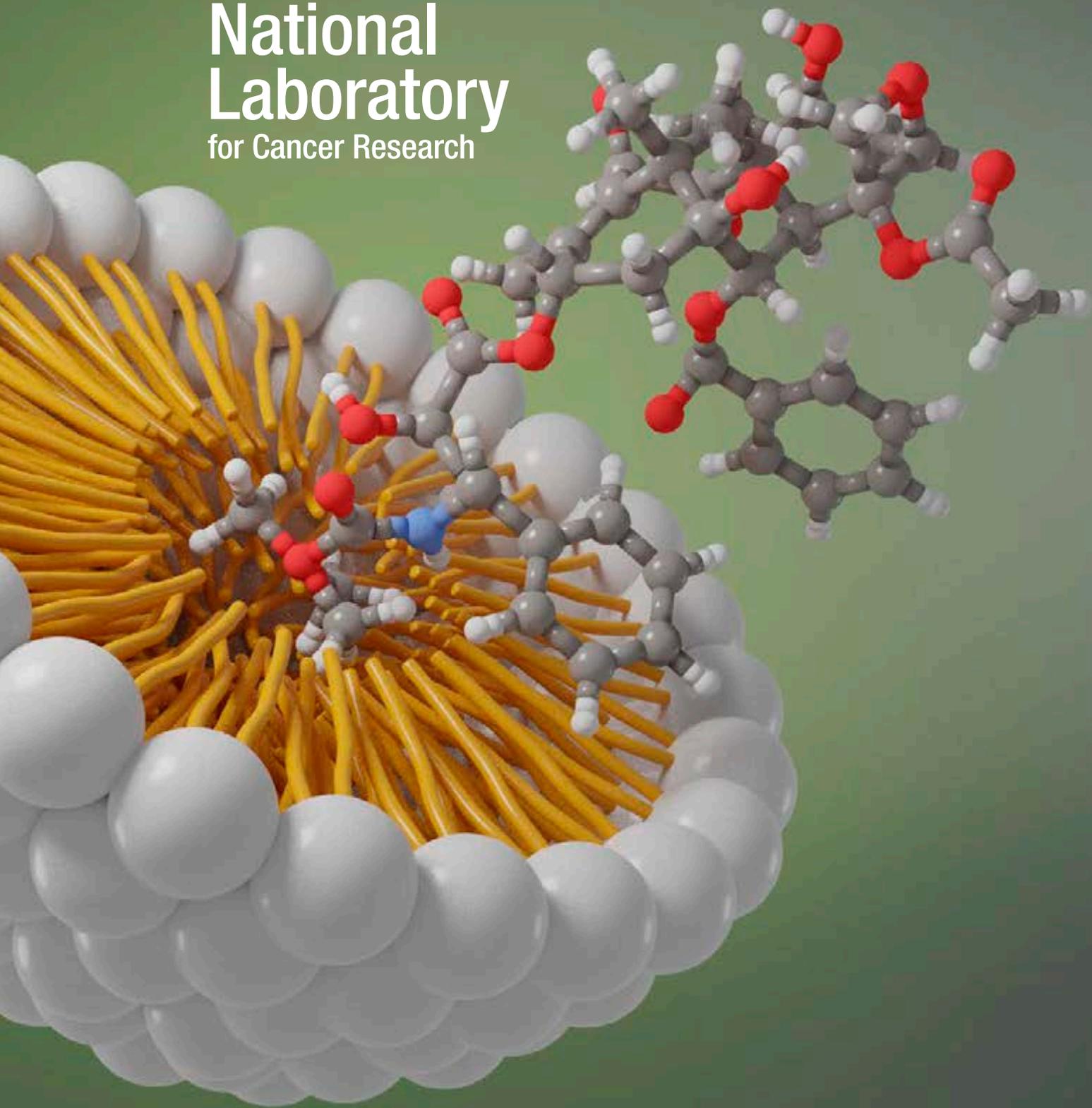
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For questions or further discussion on any of the topics highlighted here, please feel free to reach out to Dr. Dobrovolskaia via email.

Frederick National Laboratory

for Cancer Research



The NCL is part of the Frederick National Laboratory for Cancer Research (FNLCR). FNLCR is operated by Leidos Biomedical Research, Inc., for the National Cancer Institute, under contract 75N91019D00024.

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