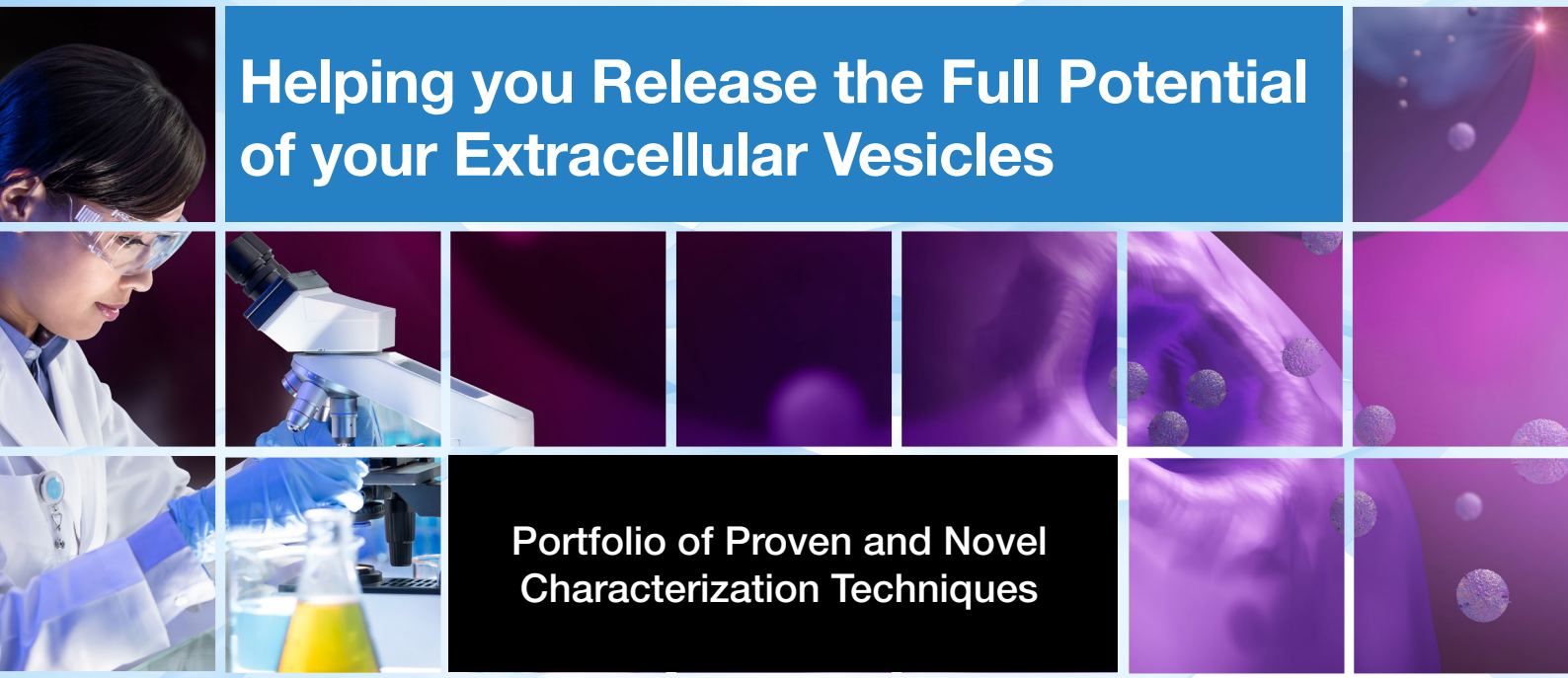
HORIBA
Scientific

Helping you Release the Full Potential of your Extracellular Vesicles



Portfolio of Proven and Novel Characterization Techniques

JOBIN YVON
Technology

EVs in Biomedicine and Biotechnology

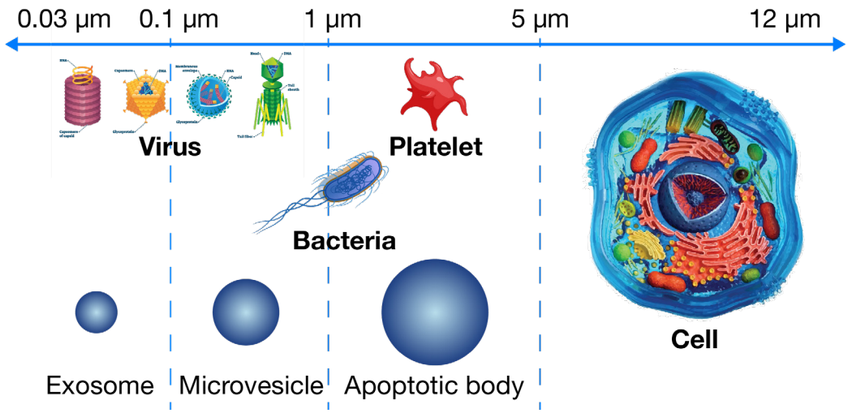
Extracellular vesicles (EVs) are organelles naturally released from cells, defined by a lipid bilayer membrane, unable to replicate (no nucleus) and containing molecules such as proteins, metabolites and nucleic acids. They act as an intracellular package delivery system for living organisms, transporting information in the form of biomolecules across long distances. They differ in their biogenesis, release pathway, content, function and size (small EVs < 200 nm, medium and large EVs > 200 nm).

Exosomes are found in many different in fluids and tissues in the body, including blood, saliva, urine, cerebrospinal fluid, and extracellular matrices, to name a few.

Extracellular vesicles are used as:

- **Biomarkers for diseases**
- **Exosome-based vaccine candidates**
- **Drug delivery systems**
- **Therapeutic entities**

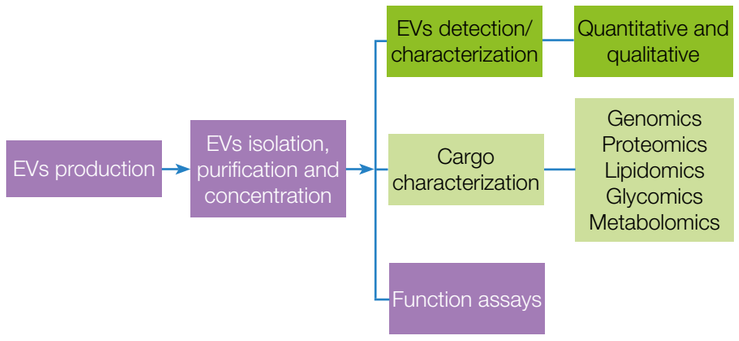
EVs convey system-wide information by delivering context-specific molecules to highly targeted (and often difficult to reach) destinations. These characteristics can be harnessed for diagnostic and therapeutic purposes. EVs serve as biomarkers, and with purposeful modification, can deliver therapeutics to hard-to-reach systems in the body.
This significant potential for commercialization has expanded interest in EVs, and many new research initiatives are focused on their diagnostic and therapeutic purposes.



Exosome size relative to other biomolecules

In the extracellular vesicles study process, HORIBA instrumentation is suitable for extracellular vesicles detection and characterization and can help EVs cargo characterization.

HORIBA tools
HORIBA helping
Other steps



EV Characterization Workflow and HORIBA Analytical Tools

EV Analysis – Complementary Techniques for Completeness

Optical, Granulometric, Morphological and Chemical Properties

The main challenge is to clearly differentiate the different types of extracellular vesicles by their size and morphology, but also by their chemical composition, their mechanical properties, and their avidity with other biomolecules allowing a better understanding/investigation of the EVs.

The International Society of Extracellular Vesicles (ISEV) recommends that EVs be characterized in multiple ways describing both physical and chemical parameters to improve the reliability and reproducibility of published results.

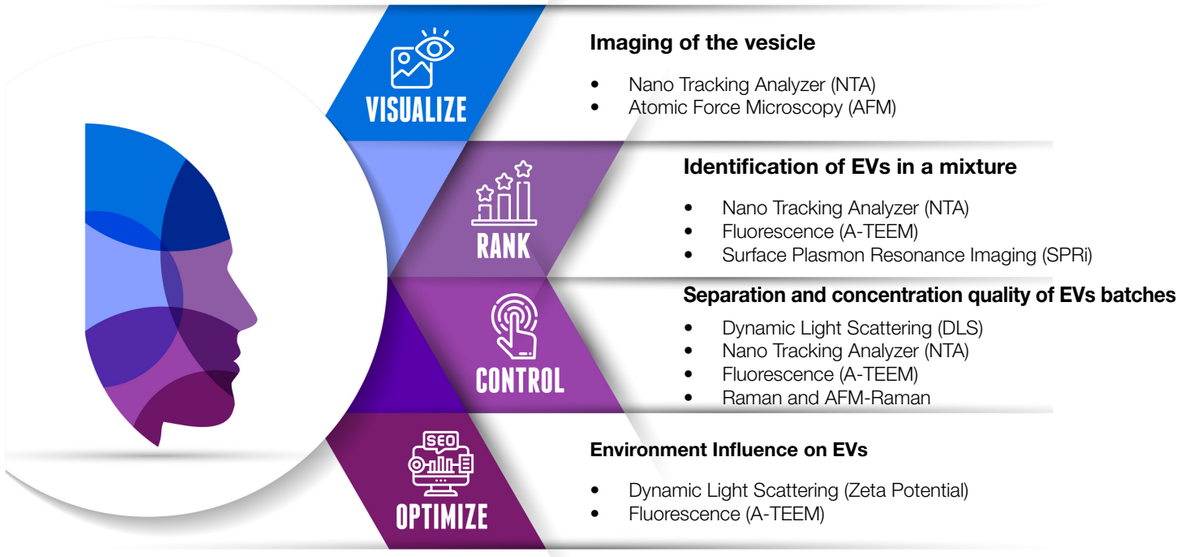
HORIBA Portfolio of EV Characterization Techniques

Physical Characterization					Molecular Profiling		
Size Distribution	**Morphology**	**Concentration**	**Mechanical Properties**	**Aggregation Evaluation**	**Chemical Composition**	**Kinetic Parameters Avidity**	**Loading**
NTA (Polydisperse Solutions) DLS	AFM	NTA Fluorescence SPRi	AFM-Raman (TERS)	Fluorescence AFM NTA DLS	Raman SERS and TERS	SPRi	Fluorescence

Visualization and characterization of EV with different techniques

ISEV recognizes that “EV characterization by multiple, complementary techniques is important to assess the results of separation methods and to establish the likelihood that biomarkers or functions are associated with EVs and not other co-isolated materials.” HORIBA has a portfolio of analytical instruments spanning physical, biochemical, and affinity characterizations that can be used to develop a comprehensive summary of EV attributes, from the lab to production, which aligns with the MISEV minimal requirements.

Modalities include: Nanoparticle Tracking Analysis (NTA); Dynamic Light Scattering (DLS); Fluorescence and the A-TEEM (Absorbance-Transmission Excitation Emission Matrix) fluorescence method; Surface Plasmon Resonance Imaging (SPRi); Atomic Force Microscopy (AFM); Raman spectroscopy; Tip-Enhanced Raman Spectroscopy (TERS); and Surface Enhanced Raman Spectroscopy (SERS). The quality of EV characterization is improved by the combination of different approaches.



Characterization of Mesenchymal Stem Cells

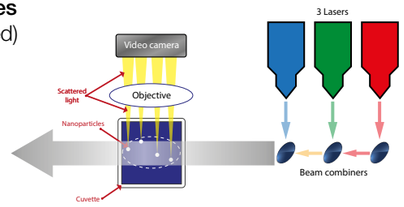
Thanks to HORIBA technologies, researchers have global information available on extracellular vesicles such as size, morphology, concentration, composition and optical properties. Below are exosomes from adipose-derived mesenchymal stem cells provided by Everzom* (France) analyzed by Nanoparticle Tracking Analysis (NTA), Dynamic light Scattering (DLS), Atomic Force Microscopy (AFM), Fluorescence, and Surface Plasmon Resonance imaging (SPRi). Exosomes were separated and purified by tangential flow filtration (FTT) and stored at -80°C before use.

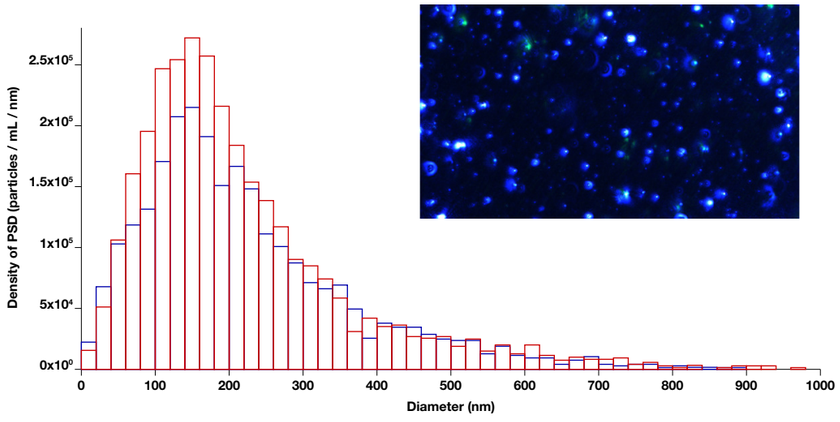
ViewSizer Multi-color NTA for Size Distribution and Concentration

The ViewSizer 3000 allows a rapid and efficient analysis of a wide range of extracellular vesicle sizes (e.g., exosomes, microvesicles, apoptotic bodies) simultaneously with high sensibility and accuracy. It includes multispectral illumination with three laser light sources (at 450 nm, 520 nm, and 635 nm). This configuration eliminates limitations of single NTA systems. The optional fluorescence mode can be useful to simultaneously identify and distinguish , tagged exosomes in a mixture.

ViewSizer 3000

- Accurate size distribution of **polydisperse samples**
- **Absolute method** (no calibration standards required)
- Particle concentration measurement
- Wide dynamic size range (10 nm – 5 µm)
- **No cross contamination**, sample cell design facilitates cleaning
- Visualize particles and processes
- **Individual particle method**





Measurement of a polydisperse sample. The duplicate measurement confirms a peak around 150 nm but shows also that bigger vesicles, beyond 300 nm, are present in the solution indicating exosome aggregation. Sizes under 100 nm indicate that vesicles fragments are also present in the solution.

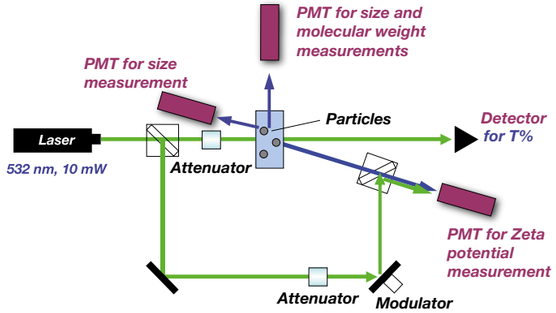
	Sample	Total counts	Mean (nm)	D50 (nm)	D10 (nm)	D90 (nm)	Estimated concentration
	Exosome 10^8 part/mL	2518	223.67	189.38	72.96	432.8	5,1x10^7 part/mL
	Exosome 10^8 part/mL	3004	217.92	178.82	76.94	418.4	6,1x107 part/mL

EVerZom

** EVerZom is a Biotech company specialized in the biomanufacturing of exosomes and working towards the democratization of regenerative medicine. Based on a patented technology and over 10 years of research in nano and biotechnologies, EVerZom is developing a proprietary industrial process that can be scaled up to produce EVs at high yield. Its process relies on a unique bioinspired physically triggered production technology enabling them to produce 10 times more EVs in 1/10th of the time compared to current methods. EVerZom aims to establish the leading European GMP-compliant exosome CDMO dedicated to exosome biotech, research institutions and big pharmas.*

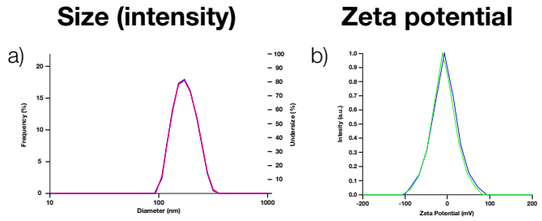
SZ-100 DLS for EV Size Distribution and Zeta Potential

Dynamic Light Scattering (DLS) is used to determine particle size distribution of a bulk sample and works best with mono-disperse size populations. It can determine the mean particle size and range for particles in the nm-µm size range. DLS is compatible with zeta potential measurements used to measure surface charge, and the two modalities are available on the SZ-100. Together they provide information on EV populations after isolation. DLS is used to validate success in separating EVs based on size, and Zeta potential is important to optimize buffer conditions that ensure colloidal stability by preventing aggregation of EVs due to their electrostatic interactions.



SZ 100V2

- EV size distribution
- EV mean **size determination**
- **Zeta potential** determination
- Quality control of EVs after isolation and concentration
- Ensure colloidal stability of EVs in suspension



Concentration	Peak (nm)	Median (nm)	Mean (nm)	Zeta potential (mV)
Exo 7.10^9 Part/mL	160.6	160.8	166.5	-6.5
Exo 7.10^9 Part/mL	160.5	160.2	166.1	-8.8

a) Size distribution and b) zeta potential of exosome stock solution (7x10^{11}part/mL) diluted 100 times in PBS (repeat measurements of the same sample). The mean of EVs size is about 166 nm and zeta potential average is -7.7 mV.

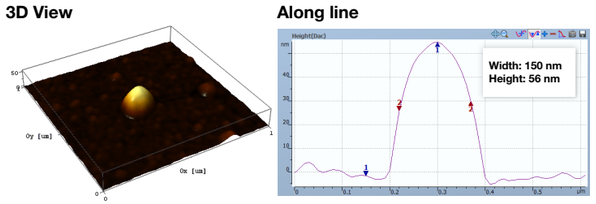
Thanks to its 3 lasers at 3 different wavelengths, the VS3000 can detect particles above 400 nm, explaining a higher average size than the one obtained with DLS. In addition, the specific design of the NTA cell with a stirring process allows the largest particles to continuously suspend in the solution.

SmartSPM AFM for Single EV Topography

Atomic Force Microscopy (AFM) is a powerful tool for the characterization of single EVs, revealing surface topology, electrical and mechanical properties of vesicles. It is used to visualize three-dimensional shape of extracellular vesicles and their surface at high resolution. EVs can be dried or evaluated in native conditions with minimal sample preparation.

Smart SPM

- **Nanoscale chemical**
- **Structural information**



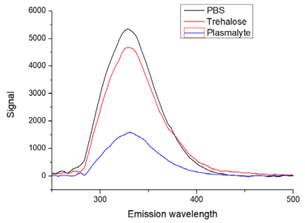
Topography of an Exosome deposited on gold substrate (dried) and characterized by AFM. The width and the height of the EV is respectively 150 and 56 nm, reflecting the dimensional changes resulting from sample preparation.

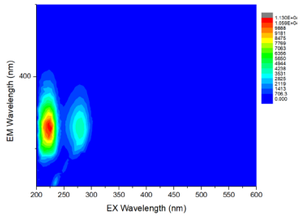
Aqualog A-TEEM for molecular fingerprinting of EVs

Absorbance -Transmission Excitation Emission Matrix (A-TEEM) fluorescence method enables rapid molecular fingerprinting and has been used to differentiate exosome types and detect contaminants. Extracellular vesicles can also be labeled with fluorophores enabling study of their biodistribution. Its potential for analysis of EVs is relatively unexplored, but it's speed, sensitivity and early data on commercial samples indicate that it may be a good tool for QA/QC. Similar to Raman and FT-IR, A-TEEM is non-destructive and label-free. Unlike these however, it is insensitive to many common excipients (like water and simple sugars) while being extremely sensitive to proteins and consequently to EVs. A-TEEM profiles are therefore better at distinguishing protein signatures in complex matrices, providing insights into composition and environment (secondary and tertiary structure). A-TEEM is a rapid, sensitive, optical technique, therefore readily deployable for process analysis or QC.

Aqualog

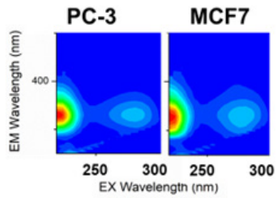
- **Unique molecular fingerprint** of EVs
- EVs phenotyping
- Sample-to-sample variation
- Aggregation evaluation
- Contaminant detection
- Molecular binding
- Solvant effect

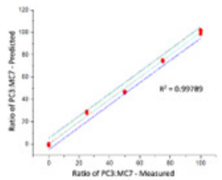




Buffer effect evaluation by A-TEEM: Exosome solution at 7x10E^8 part/mL studied in 3 different buffers (PBS, Trehalose and Plasmalyte). The decrease of intensity in Plasmalyte buffer can be due to a quenching effect between EVs and buffer.

Another example:
Unique molecular fingerprint of PC-3 and MCF7 EVs purchased commercially. A-TEEM was able to accurately predict their ratio in a mixture in a 60 second measurement



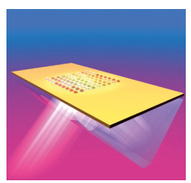


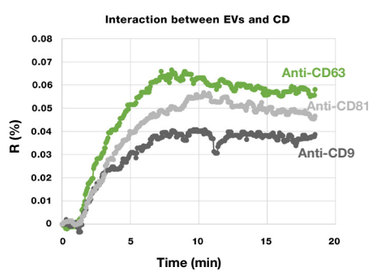
OpenPlex SPRi for Biomarker Detection

SPRi is used to detect molecular binding events in real time, determining analyte concentrations and molecular affinities. Its open architecture and imaging approach enable multiple classes of antibodies to be immobilized on the SPRi-Biochip at one time, so that the binding-related modifications to refractive index are antibody specific and can be correlated to mass variations. Surface plasmon resonance imaging is used to characterize EVs based on their membrane protein profile.

OpenPlex

- Biomolecular interaction
- **Affinity** determination
- **Kinetics** profile
- **Label-free**
- Concentration determination
- Measurement in crude media





Kinetic curves after injection of the EVs solution at 10E^9 part/mL and subtraction of the signal obtained on negative control spots.
Interaction between anti-CD63, anti-CD81 and anti-CD9 antibodies immobilized on the sensorchip and tetraspanins at the surface of targeted extracellular vesicles.

Raman Spectroscopy for chemical characterization

RAMAN spectroscopy (RS), a non-destructive and label-free technique, is used to characterize the chemical structure of the analyzed sample. This technique can be applied to extracellular characterization with diagnostic and therapeutic purpose.

LabRAM Soleil

- **Mapping** of chemical composition
- **Vesicle visualization**
- Quality control
- Purity control
- **Mechanical properties**

Raman spectroscopy can be combined with the AFM technique (TERS) for a more efficient analysis and to get nanoscale chemical and structural information and topography distribution, making the AFM-Raman platform a powerful tool.

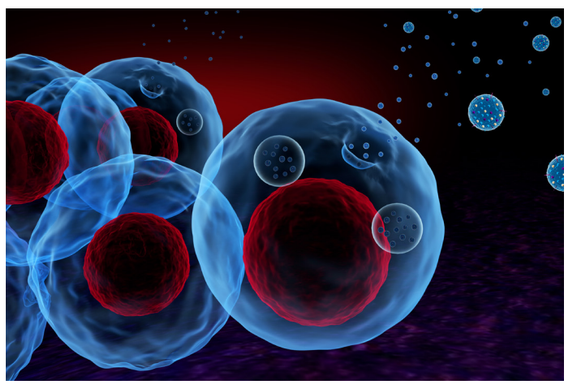
Testimonial Silvia PICCIOLINI – Labion, Don Gnocchi Institute, Italy

LABION is currently working on the biochemical characterization of extracellular vesicles (EVs) by Raman spectroscopy (RS). The team uses a HORIBA Raman spectrometer as a rapid quality check tool of EVs for in vitro or in vivo experiments and as a method to identify specific EV fingerprint used as biomarker of different diseases. The main projects on this topic are based on:

- The RS characterization of EVs isolated from the conditioned medium of human mesenchymal stem/stromal cells cultured as monolayer in vitro in serum-deprived conditions, demonstrating the ability of the method to successfully detect the main constituents of the isolated EVs (Gualerzi et al, 2017, Sci Rep; Carlomagno et al, 2021, Front Bioeng Biotechnol). The RS method was also shown to be a quick tool to assess purity of extracellular vesicle preparations and predict their functionality (Gualerzi et al, 2019, JEV);

- A label-free profiling of EVs from pro-inflammatory and anti-inflammatory microglia to identify the main bioactive constituents involved in the response to injury and in the remyelinating process occurring during neuroinflammatory disease progression. The obtained RS molecular overview gave hints to focus on the lipid compartment (Lombardi et al, 2019, Acta Neuropathol; Gualerzi et al, 2021, Neural Regen Res);

- The RS characterization of EVs isolated from the serum of patients affected by Parkinson's disease demonstrating that circulating EVs have biochemical modifications in the vesicular structure and/or cargo that are related to the disease progression (Gualerzi et al., 2019, Nanomedicine).



Contact Us

France: Tel. +33 (0)1 69 74 72 00
USA: Tel. +1 732 494 8660
Japan: Tel. +81(75)313-8121
Germany: Tel. +49 (0) 6251 8475 0
UK: Tel. +44 (0)1604 542 500
Italy: Tel. + 39 06 51 59 22 1
China: Tel. +86 (0)21 6289 6060
Singapore: Tel. +65 (6) 745-8300
Taiwan: Tel. +886 3 5600606
India: Tel. +91 (80) 4127 3637
Brazil: Tel. +55 (0)11 2923 5400
Other: Tel. +33 (0)1 69 74 72 00

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