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PROJECT FINAL REPORT

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Final publishable summary report

Executive summary

NAMDIATREAM project started on the 1st of July 2010, set for 4 years with the completion date on the 30th of June 2014. The project structure was based on four "Vertical" Technology Platforms (TPs): TP1-Fluorescent nanomaterials technologies (QD), TP2-Plasmon-optical immunodiagnostic (PlasMag), TP3-Nanotools for diagnostic and imaging based on Second Harmonic Generation (SHG), TP4-Magnetic nanowire Barcodes for disease marker detection (MagBar), and TPH-"Horizontal" platform for i) Nanoparticles synthesis, water-solubilisation, stabilisation and structural characterisation, ii) Standardisation and Risk Assessment, and iii) Preclinical validation of experimental prototypes.

NAMDIATREAM has fully completed its originally planned programme and successfully reached of all the expected deliverables and milestones.

All the technologies developed within the TPs activities have been integrated into the demonstration diagnostic systems operating on the principles of optical, plasmonic, non-linear optical and magnetic detection of cancer cells and molecular markers. Using these systems, a number of tests have been carried out on the realistic clinical samples in close cooperation with the industrial partners. The performance of the new systems proved to be comparable or superior to the quality obtained with gold standard protocols of diagnostic practice.

The project has provided a significant additional value for money for the EU stakeholders. Several technological achievements have been either carried out into a wider exploitation context or transferred into commercial settings.

Fundamental scientific achievements and breakthroughs served as the basis for several new and complementary funding opportunities targeting the advanced translational stages of the developed diagnostic systems and their expanded applications.

Public awareness of the project achievements and dissemination activities has been maintained at the very high standard through more than 450 public events in the form of meetings, publications, media coverage, trade shows and widely available web resources which have been accumulated over the project lifespan. The international and multidisciplinary dimensions of NAMDIATREAM went beyond the scope of the originally envisaged consortium activities and it has also gathered the expertise of other related projects by arranging outreach events in the fields of nanotechnology-enabled molecular imaging, therapeutics and market strategies for nanomedicine.

Educationally, the project has generated the first cohort of highly qualified experts in nanotechnology applications focused on diagnostics and monitoring of cancer. In total, four PhD and ten MSc degrees have been awarded to the students involved in the project.

Overall, the NAMDIATREAM consortium has demonstrated an outstanding commitment to achieving its challenging technological objectives along with the remarkable dissemination, exploitation outputs and training activity. This has significantly contributed to the scientific progress and technological advances in biomedical sciences along with the breakthrough clinical applications achievements in the areas of Cancer, Diagnostics, Healthcare, Nanotechnology, Nanomedicine, Smart systems for Healthcare and other relevant fields, for the ultimate benefit of the involved stakeholders.

Summary description of the project context and the main objectives

NAMDIATREAM has fully completed its originally planned programme and successfully reached of all the expected deliverables and milestones.

All the technologies developed within the Technology Platforms' activities have been integrated into demonstrator devices operating on technological innovations based on plasmonic, non-linear optical and magnetic properties for the detection of three type of cancer. Close cooperation with the industrial partners and clinical settings has enabled testing and benchmarking with clinical gold standards in order to assess the performance of the nano-enabled diagnostic devices.

WHAT HAS BEEN DEVELOPED?

NAMDIATREAM has developed cutting edge nanotechnology-based tools for multi-modal detection of biomarkers of most common cancer types and cancer metastases, permitting identification of cells indicative of early disease onset in a high-specificity and throughput format in clinical, laboratory and point-of-care devices.

WHAT BACKGROUND CONCEPTS HAVE BEEN IMPLEMENTED?

The project was built on the innovative concepts of super-sensitive and highly specific "lab-on-a-bead", "lab-on-a-chip" and "lab-on-a-wire" nano-devices utilizing photoluminescent, plasmonic, magnetic and non-linear optical properties of nanomaterials. This offered groundbreaking advantages over the existing technologies in terms of stability, sensitivity, time of analysis, probe multiplexing, assay miniaturisation and reproducibility.

WHAT WERE THE PRIMARY OBJECTIVES?

NAMDIATREAM established the following key scientific and technological objectives, which directly addressed the call topic NMP-2009-4.0-3 "Development of nanotech-based systems for molecular diagnostics and imaging".

- Produce a new generation of nanomaterials based on photoluminescence (PL) of quantum dots (QDs) as well as on plasmonic, magnetic and non-linear optical properties of nanoparticles and nanowires for subsequent use in molecular imaging and diagnostics devices,
- Functionalize the nanomaterials enabling them to recognize target molecular moieties in liquid samples and on the surface of relevant normal and diseased cells and tissues with a high level of specificity,
- Perform a rigorous characterisation of these nanomaterials to ensure the highest degree of physical and chemical quality, functional properties, manufacturing reproducibility and standardisation, following the internationally agreed guidelines by OECD,
- Establish and carry out a set of standardized risk assessment procedures aimed at identifying of biohazards and predominant exposure routes associated with the new nanomaterials,
- Develop prototype diagnostic and imaging tools incorporating the functionalised nanomaterials,
- Evaluate the performance of the prototypes in model samples and clinical diagnostic material (serum, blood, urine, biopsy specimens etc., specifically applicable to breast, lung and prostate cancers),
- Validate the manufactured devices and ensure their compliance with OECD regulations,
- Carry out technology transfer processes and to develop strategies to commercially exploit device models in collaboration with industrial partners.

ADDRESSING THE INDUSTRIAL NEEDS.

NAMDIATREAM consortium objectives involved experimental validation of nanotechnology-based medical diagnostic tools, which provided a qualitatively new level of sensitivity and accuracy in malignant disease recognition, and ensured the implementation of the developed technologies by the leading European industrial partners.

IMPACT AND BENEFIT TO THE STAKEHOLDERS.

NAMDIATREAM efforts were focused on delivering the value for money for the stakeholders through the development of super-sensitive diagnostic assays and devices allowing for the early diagnosis and monitoring of lung, breast and prostate cancers. The project has provided significant additional knowledge and breakthrough advances in cancer detection.
Several technological achievements have been either carried out into a wider exploitation context or transferred into commercial settings.

HIGHLIGHTS OF THE ACHIEVEMENTS OF EACH TECHNOLOGICAL PLATFORM

Technology Platform 1 (TP1): Fluorescent nanomaterials technologies (QD), TP Leaders – nanoGune (during Months 0-12), TCDM-TP1 (Months 13-36), and URCA (Months 37-48)

TP1 development within NAMDIATREAM project was focused towards the set of research and development goals where semiconductor fluorescent nanomaterials (quantum dots, QDs) for medical applications were synthesized, characterized, made soluble using the carefully selected procedures ensuring the best colloidal stability, functionalized in order to ensure sensitive/specific detection of genomic and proteomic biomarkers and systematically tested for their stability against the changing temperature and storage conditions.

TP1 initial research and development activity has been directed towards the development of a panel of photoluminescent QDs-tagged highly specific antibodies (Abs) and single-domain antibodies (sdAbs) enabling to detect biomarkers of breast, prostate and lung cancer (WP1-WP5). It also developed immuno-microbeads composed of polymeric microspheres encoded with different combinations of QDs and tagged with Abs or sdAbs against cancer biomarkers for their multiplexed (simultaneous) detection. Moreover, TP1 has developed diagnostic probes for ultrasensitive detection of rare disseminated and circulating tumour cells (DTC and CTC).

Ultra-small diagnostic probes based on the highly oriented conjugates of single-domain antibodies (sdAbs) against tumour biomarkers conjugated with QDs were produced, characterized and pre-clinically validated (WP8) in biological fluids and tissues utilizing flow cytometry and immunochemistry diagnostic platforms, as well as in the animal tumour models.

The **TP1 part of the project has therefore contributed into the development of safe and functionally active conjugates of nanoparticles (NP) and quantum dots (QD)-encoded microspheres (MS)** along with **clinically validated "lab-on-a-bead" and "lab-on-a-chip" prototypes employing QD and QD-encoded MS.**

TP2: Plasmon-optical immunodiagnostic (PlasMag), TP Leader - AIT

TP2 has developed a nanotechnology-based homogeneous immunodiagnostic method ('PlasMag') for the detection of diseases from in-vitro samples such as blood serum, saliva or urine (WP1 – WP5). It allows for the early detection of cancer and for the monitoring of cancer-specific biomarkers following surgery in order to check the reoccurrence of a tumour (WP8).

TP2 diagnostic approach is a fast and simple "mix-and-measure" technique. It is based on optical observation of the dynamic response of functionalized magnetic core/noble metal shell nanorods ('nanoprobes') to an externally applied time-varying magnetic field. As target molecules specifically bind to the surface of the nanoprobes, the observed dynamics of the nanoprobes changes reflecting the concentration of the captured target molecules in the sample solution can be quantified.

The **TP2 part of the project has developed a highly sensitive immunodiagnostic platform for the detection of cancer biomarkers in low volume biological samples based on the plasmon-optical properties of nanowires.**

TP3: Nanotools for diagnostic and imaging based on Second Harmonic Generation (SHG), TP Leader - UNIGE

TP3 has developed new tools for early diagnostic imaging based on the unique properties of non-centrosymmetric nanocrystals. They enhance the capability of visualizing cancer cells in human tissues,

which can greatly facilitate **diagnosis, prognosis, and therapy of malignancies**. For this goal, nanoparticles coated with specific molecules are selectively attached to cancer cells membranes and light up when illuminated. However, the optical frequencies needed to exert the optical response have a very limited penetration through skin and organ tissues and the optical response is often unstable and limited in time. TP3 approach, based on the nonlinear optical properties of nanomaterials with specific crystal structures, allows exciting the nanoparticles at any frequency, including infrared spectrum of wavelengths, which are known to penetrate much deeper into the human tissues by minimizing optical scattering. Moreover, the optical response of TP3 nanoparticles remains stable over very long time intervals (hours, days and more), allowing **long-term dynamic monitoring of disease-associated cellular structures and processes**. TP3 has also worked on adding new functionalities to the particles for using them in combination with other well-established diagnostic technologies, like MRI. The team has explored the simultaneous acquisition of multi-order nonlinear optical response both for sensing and imaging, so as to increase the specificity of detection of individual nanoparticles in spectroscopically crowded media, such as cancer cells in human blood.

The **TP3 part of the project has developed new generation robust imaging tools for spectroscopy-based cancer diagnostics in complex cancer tissues and biological samples by exploiting the nanomaterials with nonlinear optical properties.**

TP4: Magnetic nanowire Barcodes for disease marker detection (MagBar), TP Leader – TCD

TP4 was industrially driven by key partnering SMEs and multinational companies to develop and test the functionality of "lab-on-a-chip" and "lab-on-a-wire" prototype systems implementing the concept of segmented magnetic nanowires acting as barcode-type labels for detection of cancer biomarkers in biological fluids and on the surface of living cells.

The prototype system enabling the multiplexed detection of molecular biomarkers of human diseases in a high-sensitivity and throughput format using magnetic nanowires has the following major advantages over the existing and developing methods: i) it requires a low power laser for optical detection and confirmation; ii) it is not requiring any complex biochemical preparation or diagnostic procedure; iii) it requires low volumes of samples and reagents; iv) it uses miniaturised fluorescence sensors with multiple integrated narrowband filters at very low cost and complexity; v) it provides high throughput capabilities.

Successful implementation of MagBar prototype by TP4 has ensured new advancements in the development of nanoscale flow cytometry for the detection of cancer biomarkers.

TP-Horizontal – TP leaders – PUM, UCD / TCD, MPG

The "horizontal" activities of NAMDIATREAM have been focused in securing progress over the three main areas: i) quality assurance on the overall nanoprobes preparation, characterization, colloidal stability and water dispersion (pre-functional cancer labelling); ii) overall assessment of standardisation, safety and risk assessment across all platforms and iii) development of suitable in vitro representative models for nanoprobes testing towards the preclinical validation of all the four technological platforms.

NAMDIATREAM consortium has been fully committed to achieve the challenging objectives identified for our Technology Platforms (horizontal and vertical activities across the ten set work packages) and this has been reflected in its outstanding dissemination, exploitation outputs and training activities. It has significantly contributed to the scientific progress and breakthrough technological advances in biomedical and clinical sciences applicable to Cancer Research, Diagnostics, Healthcare, Nanotechnology, Nanomedicine, Smart systems for Healthcare and other relevant areas, for the ultimate benefit of the European stakeholders.

Main S&T results/foregrounds

NAMDIATREAM delivered a set of original molecular imaging and diagnostics tools introducing a number of breakthrough innovations in the following three key domains, compared to the existing state-of the-art approaches:

- Engineering of novel nanomaterial labels, utilizing their photoluminescent, plasmonic, magnetic and nonlinear optical properties, providing clear comparative advantages in sensitivity of detection methods, compared to the existing organic labels.
- Functionalizing the developed nano-labels with new types of capture molecules, including extremely stable single-domain antibodies (sdAbs) produced by llama and other highly specific primary Abs and secondary amplification Abs tagged with the developed nanolabels and optimized for the detection of rare cells attributed to breast, lung and prostate cancers.
- Developing and pre-clinically evaluating lab-on-a-bead, lab-on-a-chip and lab-on-a-wire diagnostic assays as well as biopsy imaging microscopy that combines the conjugates of novel nano-labels with innovative capture molecules along with the dedicated instrumentation for optimized readout of their photoluminescent, plasmonic, magnetic and nonlinear optical signatures.

NAMDIATREAM 22 partner project was structured four vertical and one horizontal Technology Platforms (TPs) across 10 work packages; each TP had clearly set deliverables and milestones for the development of TP1-Fluorescent nanomaterials technologies (QD), TP2-Plasmon-optical immunodiagnostic (PlasMag), TP3-Nanotools for diagnostic and imaging based on Second Harmonic Generation (SHG), TP4-Magnetic nanowire Barcodes for disease marker detection (MagBar), and TPH-Horizontal platform for i) Nanoparticles synthesis, water-solubilisation, stabilisation and structural characterisation, ii) Standardisation and Risk Assessment, and iii) Preclinical validation of experimental prototypes.

TP1: Fluorescent nanomaterials technologies (QD), TP Leaders – nanoGune (Months 0-12), TCDM-TP1 (Months 13-36) and URCA (Months 37-48)

TP1 research and development activities were based on the exploitation of key physico-chemical properties of QDs as an advanced generation of fluorescent nanoprobes, offering the following:

- Outstanding sensitivity,
- Excellent photostability,
- Multiplexed quantitative diagnostics -simultaneous detection of several tumour biomarkers.

TP1 developed a panel of photoluminescent QDs-tagged highly specific antibodies (Abs) and single-domain antibodies (sdAbs) able to detect biomarkers of breast, prostate and lung cancer. It also develops immuno-microbeads consisted of polymeric microspheres encoded with different combinations of QDs and tagged with Abs or sdAbs against cancer biomarkers for their multiplexed (simultaneous) detection. Moreover TP1 produced diagnostic probes for ultrasensitive detection of extremely rare disseminated and circulating tumour cells (DTC and CTC).

Ultra-small diagnostic probes based on the highly oriented conjugates of single-domain antibodies (sdAbs) against tumour biomarkers and semiconductor fluorescent quantum dots (QDs) were developed, characterized and pre-clinically validated in the biological fluids and tissues in the flow cytometry and immunochemistry diagnostic platforms and in the animal tumour models.

This work included:

- Isolation llama derived sdAbs with high specificity and affinity in the nanomolar range for tumour related markers including CEA, EGFR, HER2, PSMA, EpCAM and Eag1,
- Synthesis in organic phase of QDs with the quantum yield exceeding 90%, their transfer to aqueous phase by overcoating them with an amphophilic polymers ensuring high stability in biological fluids and tissues and controlled charge and number of conjugation valences on their surface,

- Highly oriented conjugation of sdAbs with QDs ensuing excellent stability, sensitivity and specificity of tumour biomarkers detection in vitro and in the animal models.

The developed diagnostic nanoprobes represent ready-to-use products for their integration into the existing multiparametric diagnostic in vitro platforms and for investigation of tumour development in the standard animal models.

The TP1 consortium has started its work from the development of the models of optimised FRET in the nano-bio hybrid materials based on the semiconductor CdSe/ZnS QDs, photosensitive membrane proteins and the antibodies tagged with the fluorescent labels. High-volume batches of highly fluorescent CdSe/ZnS and alloyed ZnCdSe/ZnS QDs were supplied to the other members of consortium for evaluation and characterization. Partner 2 (nanoGUNE) and subsequently - TCD-MED and URCA as TP1 leaders also produced high quality QD-encoded microspheres and provided this material for the development of the advanced protocols of quality control of these materials by BD Bioscience France (BD) industrial partner. TP1 leaders have also provided required materials for immunoassays development to the PGK, who was continuously involved in the biomarkers identification and characterization. Successful efforts of TP1 partners have led to the development of optimized procedures for nanoparticles transfer into the biological buffers and liquids, providing functionalized nanoparticles with the best colloidal stability and shelf life of months. This work was followed by the development of the protocols for efficient nanoparticles purification, highly oriented conjugation with the quantum dots and comparative functional activity assessment of conjugates. Developed functionally active conjugates of nanoparticles (NP) and QD-encoded microspheres (MS) were characterized ex-vivo and used for assay design towards the specific and ultrasensitive detection of genomic and proteomic biomarkers as well as for the proof-of-the concept of multiplexed FRET assays. At the next stage of the work, the lab-on-a-bead and lab-on-a-chip prototypes employing QD and QD-encoded MS were developed and pre-clinically evaluated. The final stages of the work were devoted to the development of the QD-based imaging nanoprobes for single cell cancer detection.

TP1 activities within NAMDIATREAM project were focused towards the set of research and development goals whereby semiconductor nanomaterials for medical applications were synthesized, characterized, made soluble using the carefully selected procedures ensuring the best colloidal stability, functionalized in order to ensure sensitive/specific detection of genomic and proteomic biomarkers and systematically tested for their stability against the temperature and storage.

The **TP1 part of the project has also logically developed towards generating functionally active conjugates of nanoparticles (NP) and quantum dots (QD)-encoded microspheres (MS)** as well as **the clinically validated lab-on-a-bead and lab-on-a-chip prototypes employing QD and QD-encoded MS.**

The protocols for the conjugation of full-size antibodies (Abs) and single-domain antibodies (sdAbs) to water-soluble NP and QD-tagged MS were developed and applied to preparation of the ultra-small diagnostic probes which proved to be operational in the lab-on-a-bead flow cytometry and immuno-histochemical diagnostics platforms.

TP1 leaders together with the INSERM-Marseille group have **developed functionally active conjugates of NP and QD-encoded MS** with sdAbs and also "**Lab-on-a-bead" and "lab-on-a-chip prototypes" employing QD-sdAbs conjugates and QD-encoded MS.**

Ideal diagnostic nanoprobes should not exceed 15 nm in size and should contain high-affinity homogeneously oriented capture molecules on their surface. An advanced procedure for the partial reduction of antibody (Ab) was used to cleave each Ab molecule into two functional half-Abs, 75-kDa heavy–light chain fragments, each containing an intact antigen-binding site (Figure TP1.1, left). Affinity purification of half-Abs followed by their linkage through their free sulfhydryl groups to quantum dots (QDs) yielded uniformly oriented QD–Ab conjugates whose functionality was considerably improved compared to those obtained using the standard protocols.

Ultrasmall diagnostic nanoprobes were engineered through oriented conjugation of QDs with 13-kDa single-domain Abs (sdAbs) derived from llama IgG. sdAbs were tagged with QDs via an additional cysteine residue specifically integrated into the C-terminal region of sdAb using genetic engineering. This approach made it possible to obtain sdAb-QD nanoprobes <12 nm in diameter comprising four copies of sdAbs linked to the same QD in an oriented manner (Figure TP1.1, right). sdAb-QD conjugates against carcinoembryonic antigen

(CEA) and HER2 exhibited an extremely high specificity in flow cytometry, and the quality of immunohistochemical labelling of biopsy samples was found to be superior to that of labelling according to the current "gold standard" protocols of anatomo-pathological practice. The nano-bioengineering approaches developed can be extended to oriented conjugation of Abs and sdAbs with different semiconductor, noble metal, or magnetic nanoparticles.

Finally, all necessary protocols for the conjugation of full-size antibodies (Abs) and single-domain antibodies (sdAbs) to water-soluble NP and QD-tagged MS are developed and applied to preparation of the ultra-small diagnostic probes which proved to be operational in the lab-on-a-bead flow cytometry, in immunohistochemical diagnostics platform, and also in the animal models *in vivo* (see below).

One of the principal objectives of the TP1 within the consortium was to assess the suitability of nanoprobes as diagnostic tools in oncology. Therefore, tissues and biological samples of metastatic tumour mouse models were collected and used **for the validation of these nanoprobes to detect primary tumours as well as disseminated tumour cells** in the models utilizing two metastatic breast tumour cell types.

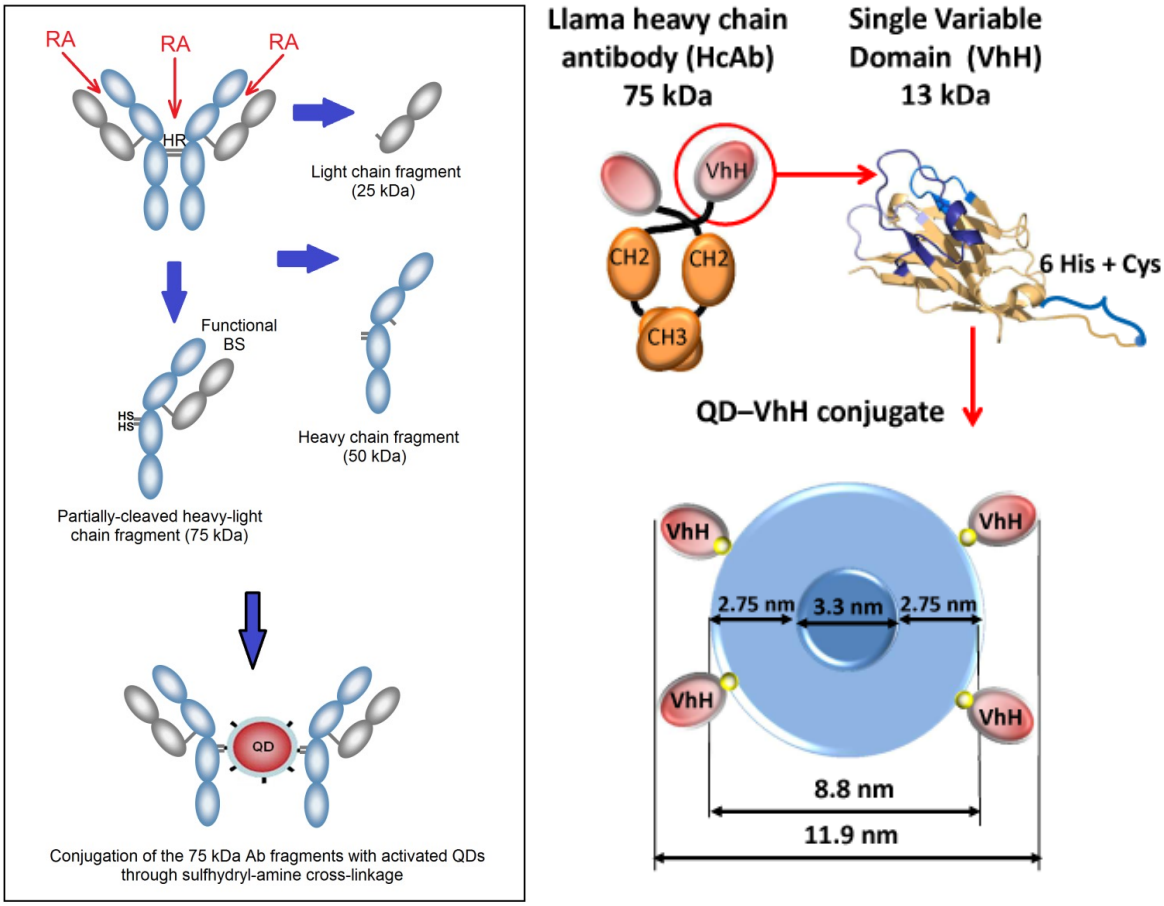


Figure TP1.1. Strategies for oriented conjugation of the full-size (left) and single-domain (right) antibodies with the semiconductor nanocrystals quantum dots.
Left: The antibody (Ab) reduction with the reducing agent (RA) DTT or 2-MEA and the strategy of conjugation of the reduced Abs with QDs. The Ab fragments that may be obtained after the disulfide bond cleavage and according to different reduction protocols are the Ab light chain fragment, Ab heavy chain fragment and partially cleaved Ab heavy–light chain fragment. In the last case, only the disulfide bonds of the Ab hinge region (HR) are cleaved and this product, in contrast to two first products, where the heavy and light Ab chains are completely separated, the Ab binding site (BS) composed of different parts of the Ab heavy and light chains is intact and functionally active. The conjugation of SMCC-activated QDs with Ab fragments generates nanoprobes with a highly improved functionality of ligand-specific recognition and binding. All objects are shown to scale.
Right: The "anatomy" of an ultrasmall nanoprobe engineered from genetically modified single-domain antibodies and semiconductor CdSe/ZnS quantum dots and the result of immunohistochemical application of this nanoprobe to clinical diagnosis using biopsies. Schematic representation of the smallest functional llama antibody fragment (a single-domain antibody (sdAb or VhH)) and an ultrasmall nanoprobe engineered from a CdSe/ZnS QD and sdAbs via sdAb oriented

conjugation through the His–Cys linker. sdAbs were C-terminally fused to a hexahistidine tag for the detection and purification purposes, and an additional C-terminal free cysteine residue (blue) was introduced for specific site-directed, oriented conjugation of sdAbs with the quantum dot. The sdAb antigen-binding loops are shown in blue; the sdAb structure was adapted from the PDB structure.

In order to obtain metastatic spread of human breast tumour cells in mice, HER2-overexpressing human SK-BR3 breast cancer cells were injected intra-hepatically in mouse embryos. After 3-5 months the mice developed primary tumours in the liver and metastases in the lung, testes, mesenterium and brain. In addition to organs, we have received bone marrow, blood and ascitic fluid samples from these mice to validate *ex vivo* engineered nanoprobes, e.g. anti-HER2 sdAb conjugated with QDs (HER2-QD) as diagnostic tools to detect tumour cells. Protocols were established for fixation of blood smears, cytospins and tissue slides of biological samples prior to immunostaining with nanoprobes. Analysis was performed by advanced microscopy methods, including confocal and multi-photon fluorescent microscopy. Tumour tissues and biological samples from tumour bearing mice that had been orthotopically injected with HER2-negative human MDA-MB-231 cells into the mammary gland were used as negative controls. Prior to tumour cell injection all breast tumour cells were assessed for their HER2 expression by Western Blot and/or flow cytometry.

A second metastatic breast cancer model was established, the BT-474 model. Here, the BT-474 human breast cancer cells were injected into the mammary gland of nude mice; additionally a hormone pellet was implanted into the neck of each mouse in order to support the metastatic spread of the tumour cells (Figure TP1.2). Three months after injection, tumours developed and mice were sacrificed. Primary tumours, lymph nodes, organs, bone marrow and blood smears were collected, in order to validate *ex vivo* engineered nanoparticles e.g. anti-HER2 sdAbs conjugated with QDs (HER2-QD) as diagnostic tools to detect tumour cells. As shown in Figure TP1.2 a primary mammary BT474 tumour developed in the mouse, accompanied by metastatic spread of tumour cells in lymph nodes and the lung.

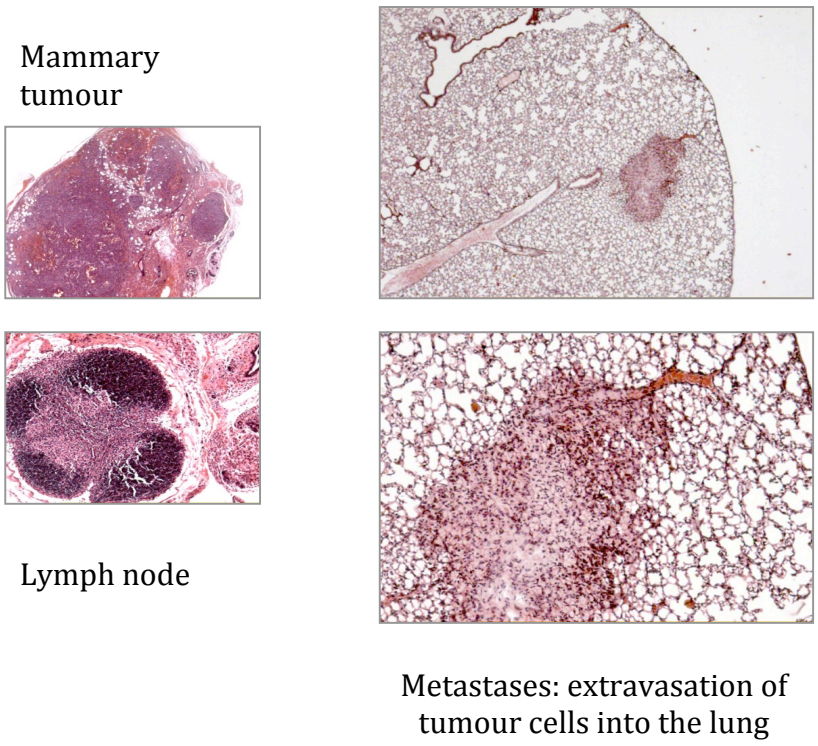


Figure TP1.2. Metastatic mammary tumour model of BT-474 cells, orthotopic tumour model.

Furthermore, a protocol was established to depict the minimum amount of tumour cells by the use of QD-HER2 nanoprobes in cytospins, containing different amounts of human HER2 overexpressing tumour cells and monocytes collected from a human donor.

Specific binding of an anti-HER2 sdAbs conjugated with QDs (HER2-QD) was further confirmed in comparison to control QD in biological samples of the humanized metastatic SK-BR3 breast tumour mouse model. The unconjugated QDs (QD) resulted in a weak unspecific background staining (Figure TP1.3, lower panel).

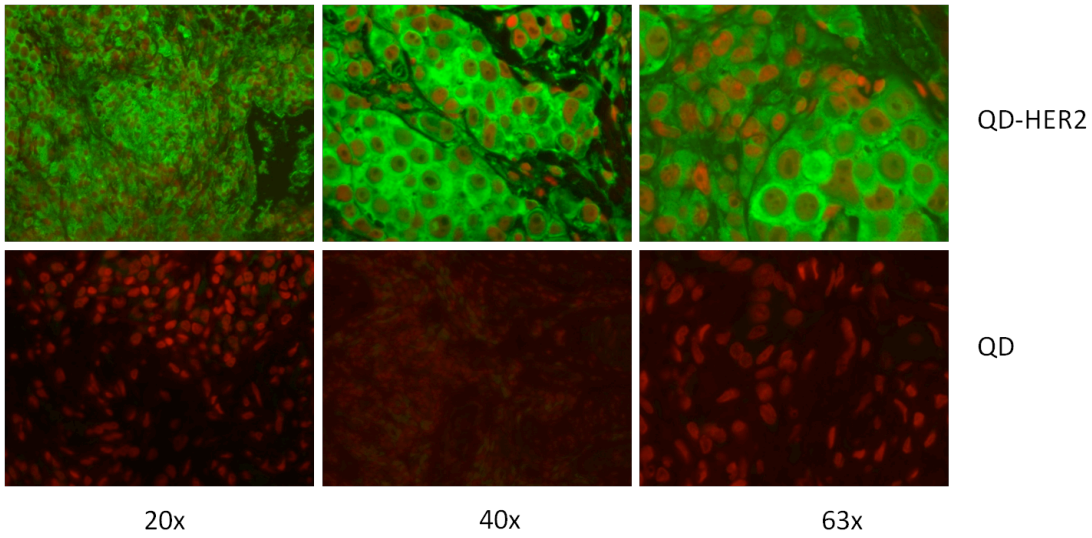


Figure TP1.3. Microscopic images show staining for HER2 protein expressed in tumour cells that invaded the testes. Sections of testes were obtained from the humanized metastatic SK-BR3 breast tumour model. Non-conjugated QDs (QD) were used as negative control (lower panel) and anti-HER2 single domain antibody conjugated with quantum dots (QD-HER2, upper panel) were used for the staining of HER2 expressed on tumour cells. In green, tumour cells were detected by QD-HER2 and in red, nuclei were stained using DRAQ5. In comparison to conjugated QDs, unconjugated QDs only resulted in minor unspecific staining of cells. Three different magnifications are shown. The wavelength excitation used for single domain antibody conjugated to HER2 was 405 nm, and for DRAQ5 was 633 nm. Mounting medium: Cytoseal 60. Same exposure times were used for all tissues stained with either QD or QD-HER2 and for all magnifications.

The nanoprobes were then validated in HER2 overexpressing breast tumour xenografts (BT-474 model) using QD-HER2. Here, Tumour material from the metastatic BT-474 breast tumour model was collected and used for the validation of QD-HER2 staining. Specific binding of anti-HER2 single-domain antibody conjugated with quantum-dots (HER2-QD) was confirmed in comparison to control QDs in biological samples of the metastatic breast tumour mouse model. Tumour cells overexpressing HER2 were detected in the primary mammary tumour using QD-HER2. Tumour cells metastasized into lymph nodes and lung were detected in tissues using QD-HER2 as well. In comparison, the unconjugated QDs (QD) demonstrated in a weak nonspecific background staining (Figure TP1.4, right image).

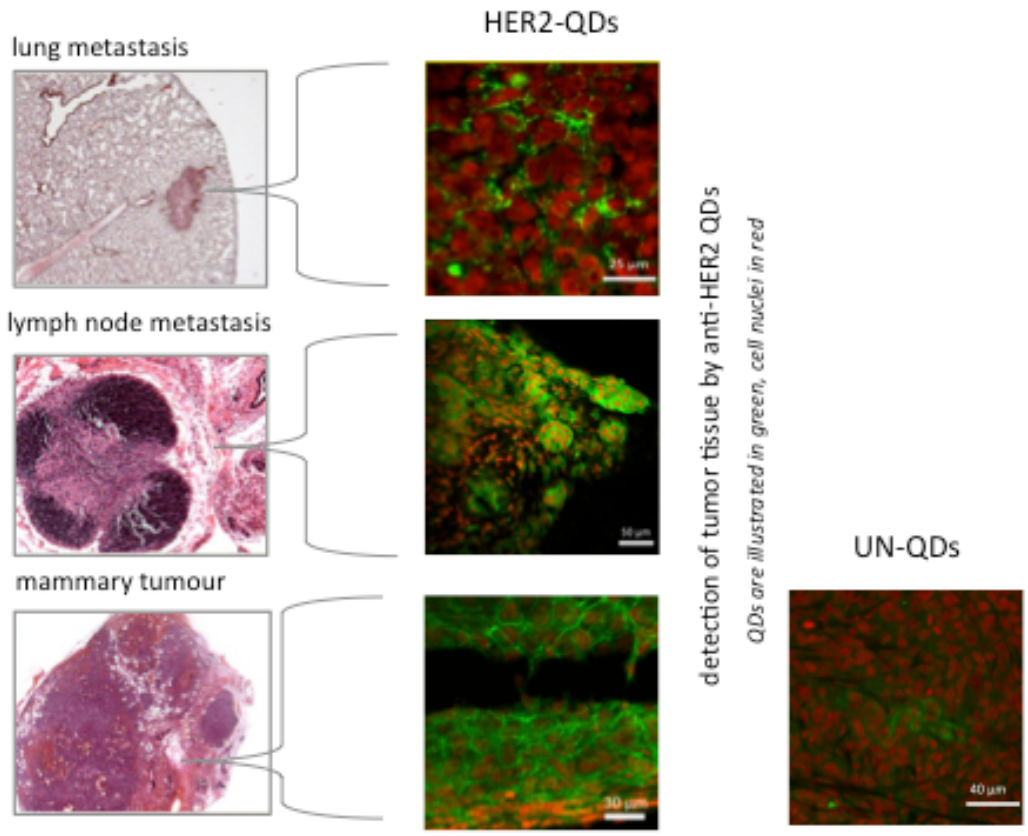


Figure TP1.4. Detection of breast cancer cells in a metastatic BT-474 mouse model. Tumour cells were determined by H&E staining und by detection of QD-HER2 that bind specifically to tumour cells expressing HER2 protein.

To summarise, the industrial outcomes of TP1 include:

- Diagnostic assays for breast, prostate and lung cancer using solid-state chips with the ultra-small nanoprobes based on highly orientated conjugates of single-domain antibodies and fluorescent semiconductor nanocrystals,
- Diagnostic assays for breast, prostate and lung cancer using liquid-state chips using microspheres optically encoded with the combinations of semiconductor quantum dots of different colours. This provides the potential to study various key cell-identifying sites using quantum dots with separate emission signals so that the resulting information can be multiplexed.

These outcomes are organically based on the key advantages offered by the products developed by TP1, namely:

- Outstanding sensitivity: the nanoprobes are at least 10-time brighter and have also better affinity due to the possibility to have the sdAbs recognition sites to be oriented in the same manner and to be open for interaction with the antigens,
- Excellent photostability: the developed nanoprobes are at least 100-time more photostable,
- Multiplexed quantitative diagnostics: simultaneous detection of several tumour biomarkers.

TP2: Plasmon-optical immunodiagnostic (PlasMag), TP Leader - AIT

TP2 has developed a **nanotechnology-based homogeneous immunodiagnostic method ('PlasMag') for the detection of disease markers in *in-vitro* samples such as blood serum, saliva or urine**. It allows the **early detection** of cancer and the monitoring **of cancer-specific biomarkers** following surgery in order to check for reoccurrence of a tumour.

TP2 diagnostic approach is a fast and simple "mix-and-measure" technique. It is based on optical observation of the dynamic response of functionalized magnetic core / noble metal shell nanorods ('nanoprobes') to an externally applied time-varying magnetic field. As target molecules specifically bind to the surface of the nanoprobes, the observed dynamics of the nanoprobes changes, and the concentration of target molecules in the sample solution can be quantified. The detection principle is illustrated in the Figure TP2.1 and explained in detail in the figure caption.

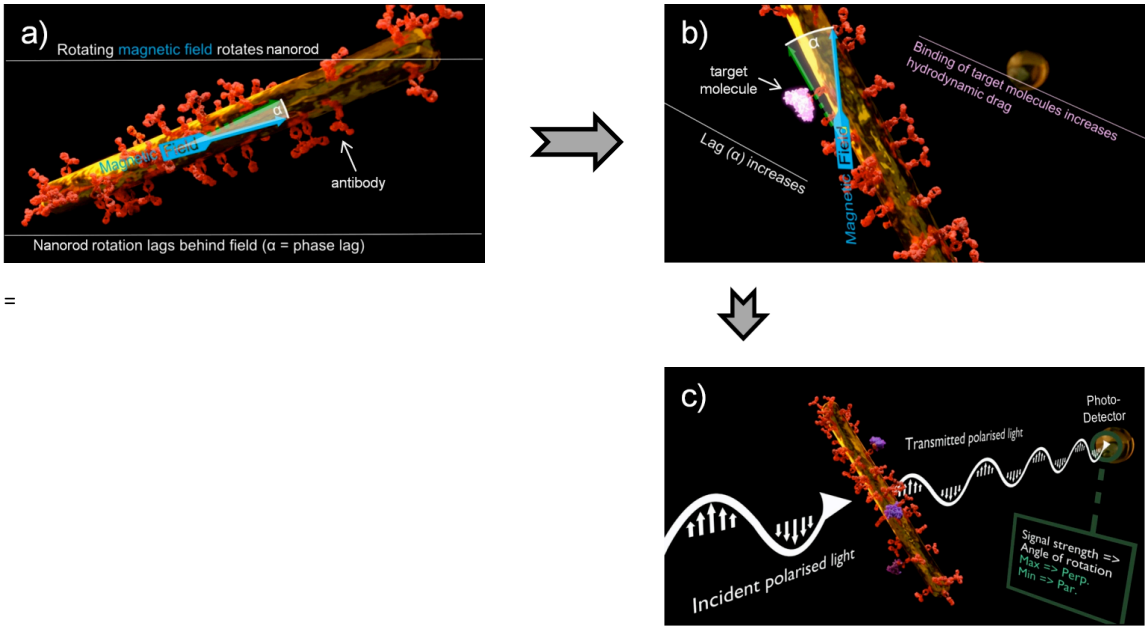


Figure TP2.1. PlasMag concept.

a) Antibody-functionalized magnetic core / noble metal shell nanorods (nanoprobes) are mixed with the sample fluid and excited by a rotating magnetic field (RMF). Due to hydrodynamic drag in the sample fluid, the nanorods follow the RMF with a phase lag angle α.

b) As target molecules bind to the nanoprobes, the hydrodynamic drag and, consequently, α increases. Thus, α presents a direct measure of the average number of target molecule bound to the nanoprobes.

The phase lag angle α is measured optically in polarized light.

During the NAMDIATREAM project, TP2 has developed the PlasMag diagnostic concept, from an idea to the technology,that has been eventually validated by a prototype instrument. This involved a number of breakthroughs advances in the enabling technologies, namely:

- Establishing theoretical models that describe nanoprobe dynamics in a rotating magnetic field and allow the average molecular shell layer thickness (i.e. the level of analyte binding) of the nanoprobes to be deduced directly from data fits to the measured phase lag spectra,
- Synthesis of patented PlasMag nanorods comprising a continuous thin noble metal shell layer system that provides long-term stability of the magnetic core against degradation in aqueous buffer solutions,
- Transfer of the core-shell nanorods from their original organic solvents to aqueous buffer solutions by coating with an amphiphilic polymer and their single-particle long-term charge stabilization against agglomeration under physiological salt conditions,

- Developing and optimizing two generations of PlasMag measurement devices (laboratory device & prototype instrument) to optically measure the dynamics of nanoprobes in rotating magnetic fields. Here, the prototype resembles a transportable and automated device with improved sensitivity,
- Direct microscopic characterization of nanoprobe reorientation in magnetic fields by a modified single particle tracking microscope,
- Proof-of-concept of the PlasMag detection technology by fast homogeneous detection of the breast cancer biomarker HER2 level directly in the sample solution,
- Development of a positive read-out system for the sensitive detection of the small molecules cortisol and neopterin and transfer of the system to a lateral flow dipstick format for direct point-of-need measurements from whole blood samples.

Below, the specific technological achievements are described in more detail.

Specific scientific and technological results achieved

System modelling

An accurate description of the dynamics of nanoprobes excited by externally applied time varying magnetic fields requires solving the Fokker-Planck equation, which can only be accomplished by numerical simulations. We determined a set of empirical equations for nanoprobes excited by rotating magnetic fields from numerical solutions of the Fokker-Planck equation, which can now be routinely applied to fit the experimentally obtained data (phase lag spectra). Since all other parameters that are relevant for the data fit can be measured independently, our empirical model allows us to directly deduce the most relevant factor of our measurement method, which is the mean molecular shell thickness of the nanoprobes (i.e. the value that changes on analyte binding). Therefore, the model is a very powerful tool for characterizing nanoprobe dispersions and their corresponding rotational dynamics.

Nanoprobes (nanorod synthesis, solvent transfer, stabilization and functionalization)

TP2 molecular detection method requires rod-shaped nanoprobes consisting of a magnetic core for alignment control by an external magnetic field and a noble metal shell for oxidation protection of the magnetic core as well as amplification of the optical nanoprobe signal, which can be achieved by exciting localized surface plasmon resonances in the noble metal shell. Both goals can be achieved by choosing gold (Au) as noble metal shell material.

In terms of size, size distribution, magnetic properties and scalability of fabrication, cobalt (Co) nanorods synthesized by an organometallic approach do present the best base material for the desired nanoprobes. However, as Co and Au are immiscible, direct deposition of Au onto the Co nanorod cores does not lead to conformal noble metal shell growth, but results in Au nanoparticles assembled onto the nanorods with minimal contact area to the Co core. To overcome this challenge, TP2 has introduced an intermediate buffer layer, which allows subsequent covering by noble metals. This patented noble metal shell layer system provides the best protection of the core against oxidation (see Figure TP2.2).

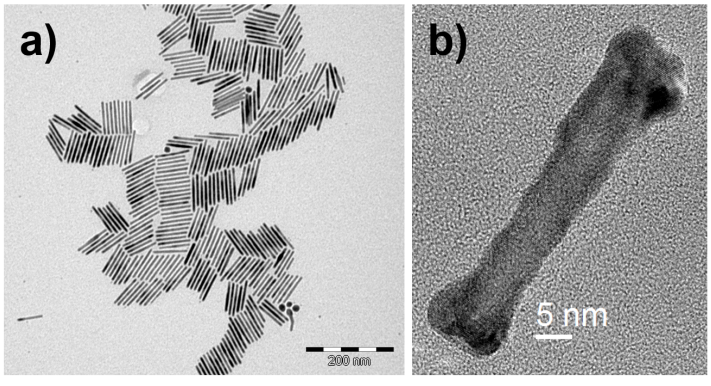


Figure TP2.2. Tunnelling Electron Microscopy (TEM) images of
a) plain Co nanorods
b) Co-nanorod after synthesis of the noble metal shell layer system.

Following synthesis, the nanorods are usually stabilized by hydrophobic surfactants in organic solvents such as toluene, so they have

to be transferred and stabilized in aqueous solutions, first. To that end, TP2 in conjunction with TP-H have developed an approach that relies on coating the nanorods by an amphiphilic polymer, i.e. a polymer comprising hydrophobic side chains for the linkage to the nanorod surface and a hydrophilic backbone that provides water solubility through charged groups. Excellent stability of the resulting nanorod dispersions in buffers with physiological salt conditions has been verified by their highly negative Zeta potential. Following water stabilization, the nanorods are functionalized by conjugating antibody recognition agents to the carboxyl groups of the polymer backbone using EDC / Sulfo-NHS carboxyl activation.

Devices (laboratory setups and prototype)

TP2 has initially built a versatile laboratory setup of the PlasMag device comprising different measurement modes and parameter ranges. It can be operated both in transmission and scattering modes and supports quasi-continuous sweeps of both the magnitude and frequency of the applied rotating magnetic field. In addition, a reference setup using magnetic fluxgate detection of nanoparticle relaxation has been established which allows direct comparison to the PlasMag device results. Furthermore, the fluxgate reference setup also enables detection of nanoparticle dispersions without optical anisotropy.

Based on the experience gained from operating the laboratory instruments, we also built an advanced second-generation prototype instrument with reduced complexity and costs that supports automated operation and is also easily transportable. The prototype consists of a desktop measurement box containing the coil system to generate the rotating magnetic field (RMF) and the optics to measure the dynamic reaction of the nanoprobe dispersions to the RMF. The entire system is computer-controlled by a LabVIEW program running on a laptop computer, and the power supplies and capacitor switches for driving the RMF are situated in a separate power supply box. Figure TP2.3 shows images of all three realized devices.

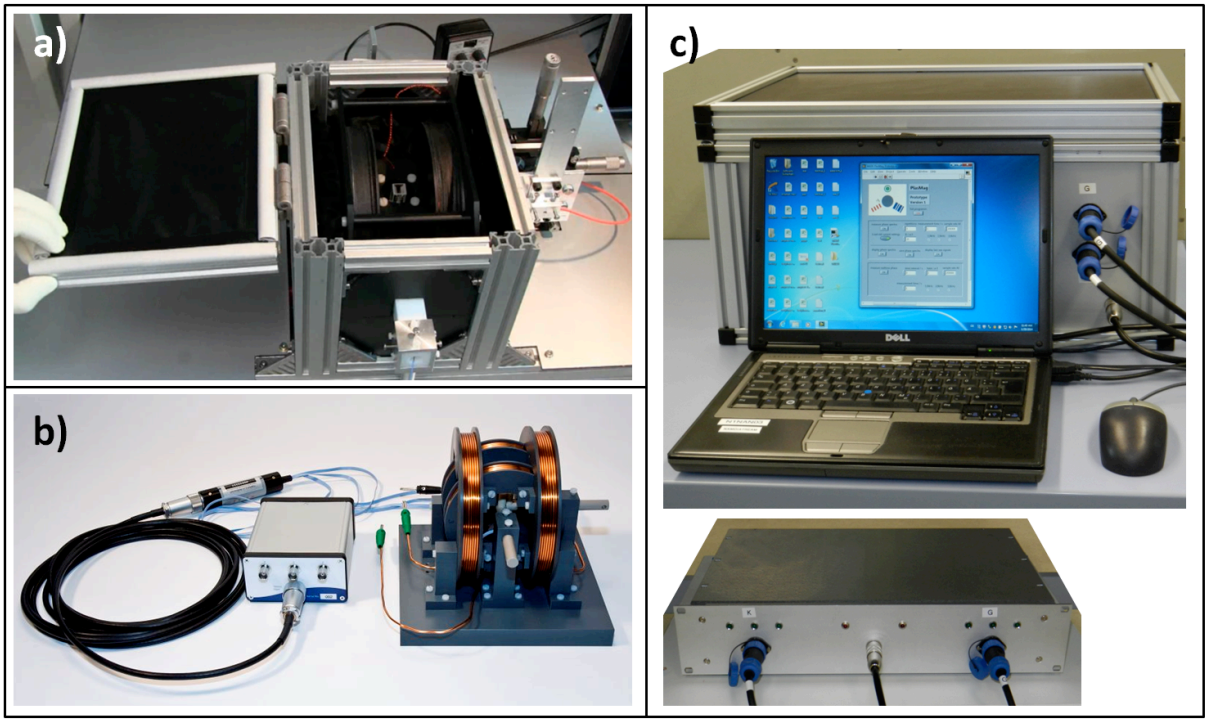


Figure TP2.3: Overview of the different realized measurement devices: a) PlasMag laboratory device (only core measurement box, not shown are the coil power supply, the lock-in amplifier, the computer and the laser controller). b) Fluxgate reference device (not shown are the coil power supplies and the computer). c) PlasMag prototype device, consisting of the measurement box, the power supply and a laptop computer (all parts shown).

In addition to the devices for measuring average nanoprobe relaxation properties, TP2 has developed a prototype instrument for microscopic observation of single nanoprobe relaxation in externally applied time-

varying magnetic fields. With this aim, a NanoSight NS500 system has been refitted with a magnetic field unit that allows aligning dispersed nanorods in the field direction. Furthermore, a spinning polarization filter wheel has been added to observe both polarization directions of the scattered light simultaneously. This functionality allows TP2 to experimentally determine the aspect ratio of individual particles, which is beneficial to identify the quality of the particle dispersion (i.e. single particle versus agglomerations).

PlasMag validation

Validation of the PlasMag principle has been carried out by detecting the soluble form of the breast cancer biomarker HER2 (sHER2). For this purpose, nanorods were functionalized with the monoclonal antibody Herceptin and characterized the molecular shell properties of different nanorod dispersions in buffer by fitting measured phase lag spectra to our empirical model. TP2 data fits result in reasonable added molecular shell thicknesses of about 15 nm for the antibody functionalization and another 10 nm for the antigens. These results demonstrate that the PlasMag detection principle is both theoretically well supported and experimentally feasible.

For actual assay experiments, it is sufficient to detect the relative phase lag difference of nanoprobes exposed to the sample in comparison with the nanoprobes immersed in a reference solution at a single magnitude and frequency of the applied RMF. Figure TP2.4 shows the resulting phase lag differences for varying concentrations of spiked sHER2 antigen both in buffer and in serum.

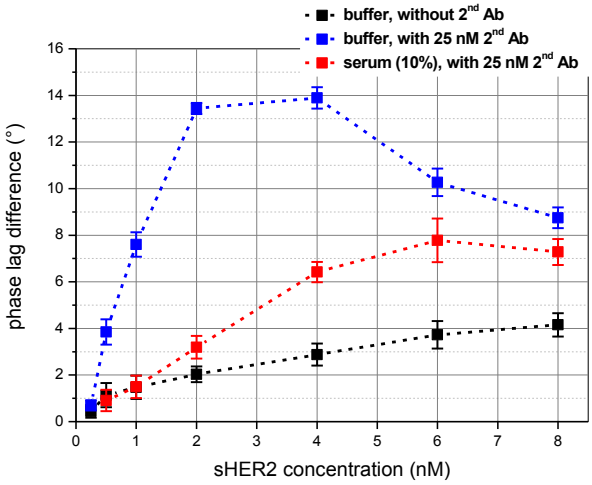


Figure TP2.4: Herceptin-sHER2 assay results.
Plotted are the signals (measured phase lag differences to a reference sample) across the spiked sHER2 concentration.

- Black data: Buffer, only sHER2 added, LoD ~ 500 pM
- Blue data: Buffer, sHER2 & 2nd Ab added, LoD ~ 280 pM
- Red data: Serum (1:9 dilution with buffer), sHER2 & 2nd Ab added, LoD ~ 500 pM (corresponds to 5 nM for undiluted serum)

Parameters

- Buffer: 10 mM HEPES, 150 mM NaCl, 0.05% v/v Tween 20, pH 7.4
- RMF excitation: 1kHz, 10 mT
- Nanoprobe concentration: 10 pM
- Secondary antibody (2nd Ab): R&D Systems, AF1129, fixed conc. (25 nM)

In buffer solutions, the black line shows the phase lag increases caused by binding of the sHER2 antigen alone, while the blue line corresponds to a fixed concentration (25 nM) of secondary antibodies added together with the analyte. By adding secondary antibodies, the measured change in hydrodynamic nanoprobe volume on binding of the sHER2 analyte increases in proportion to the amount of bound analyte alone, thus causing higher measured signals and, consequently, an improved limit of detection (LoD) by about a factor of 2 (LoD determined as the analyte concentration for which the signal reaches 2.5x the

standard deviation). The decrease of the measured phase lag difference at higher analyte concentrations is due to the decreasing ratio of analyte molecules/secondary antibodies.

In serum, nonspecific adhesion of proteins to the nanoprobes makes such measurement method rather insensitive to direct detection of analyte molecules (measured phase lag differences within the standard deviations, data not shown). However, this problem can be overcome by parallel addition of secondary antibodies, which results in a systematic increase of hydrodynamic nanoprobe volume with analyte concentration (red data in Fig. TP2-4). In 10% serum (diluted 1:9 with buffer, serum from a pool of healthy male individuals), a LoD of ~500 pM for spiked sHER2 is reached, which corresponds to ~5 nM for undiluted serum. While this is still a factor of about 40 higher than the clinical cut-off concentration of sHER2 in serum (~140 pM), by further enhancing the properties of our nanoprobes (e.g. their optical extinction signal or the homogeneity, hydrodynamic size and activity of the antibody immobilization) as well as the assay conditions (e.g. application of labelled secondary antibodies and additional binding of large molecular weight biopolymers to enhance the signal even further), it is fully feasible that the PlasMag method can be optimized to be applicable for direct monitoring of soluble cancer biomarkers in serum samples.

Small molecule Apposition System

TP2 successfully achieved a positive read-out system for detecting the small molecules cortisol and neopterin. These systems circumvent the inability of most small molecules to directly bind a secondary signalling antibody by modifying the primary antibody with a binding moiety close to the analyte binding site. In case the analyte binds, this binding moiety is still accessible to a secondary reporter molecule, but gets sterically hindered by added blocker molecules that bind to the primary antibody in case the analyte is not present. This way, positive read-out immune-assays for small molecules are established, and we have been able to demonstrate detection limits for cortisol and neopterin of ~5 ng/ml for ELISA-type assays.

Furthermore, pre-clinical validation work has been carried out, transferring the small molecule positive read-out systems to lateral flow dipstick formats using gold nanoparticles as specific probes. TP2 demonstrated positive readout immuno-sensing of the small molecule cortisol directly from serum, whole blood and saliva within minutes (spiked samples and clinical samples). These results present an important step towards a fast and sensitive point-of-need detection of biomarkers for the detection and monitoring of inflammation and cancer.

Expected industrial outcomes of TP2 include:

- **Nanotechnology-based homogeneous immunodiagnostic method ('PlasMag'),**
- **Detection of diseases from biological samples such as blood serum, saliva or urine**,
- **Early detection** of cancer biomarkers,
- Monitoring **of cancer-specific biomarkers,**
- Competitive cost per sample,
- User-friendly technology.

TP3: Nanotools for diagnostic and imaging based on Second Harmonic Generation (SHG), TP Leader - UNIGE

TP3 developed **nanotechnology-based tools that** allow **in depth imaging** of healthy and cancer-affected tissues by using non-centrosymmetric nanoparticles developed and validated within the project. Currently, three nanoproducts based on non-centrosymmetric materials have been **licensed and commercialised** by TIBIO Sagl.

Screening of physico-chemical, optical, magnetic, and biocompatibility properties of several nanomaterials with nonlinear optical response

This accomplishment has been directly related to the first TP3 deliverable of the project, aimed at the translation from the initial research standpoint (*existence of an efficient interaction mechanism allowed by multiphoton microscopes with a defined family of nanomaterials not yet exploited*) to the actual implementation of these nanoparticles probes for bio-labelling and imaging, by selecting the best candidates against several properties (optical efficiency, stability in physiological environment, biocompatibility and colloidal stability).

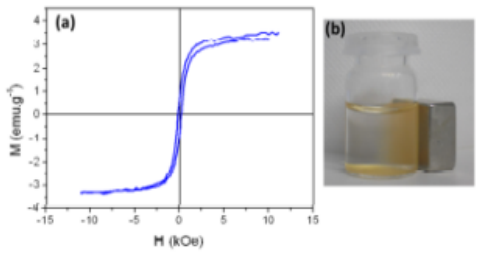
TABLE 1 **Hyperpolarizability $\langle\beta_{np}\rangle$, Normalized Hyperpolarizability $\langle d\rangle$, and Normalized Hyperpolarizability from Bulk Materials Found in Literature $\langle d_{lit.}\rangle$ of $KNbO_3$, $LiNbO_3$, $BaTiO_3$, KTP, and ZnO Nanocrystals**

nanocrystal	$\langle\beta_{np}\rangle \pm \Delta\beta$ ($\times 10^{-24}$ esu)	$\langle d_{np}\rangle \pm \Delta d$, pm/V	$\langle d_{lit}\rangle$, pm/V
$KNbO_3$	7.2 ± 2.4	3.4 ± 1.1	15.3
$LiNbO_3$	21 ± 7.0	4.8 ± 1.6	17.3
$BaTiO_3$	11.0 ± 3.5	4.6 ± 0.7	14.1
KTP	25.8 ± 6.0	1.4 ± 0.3	7.6
ZnO	9.6 ± 2.8	1.9 ± 0.6	2.8

TABLE 2 **Cytotoxic Effect after 5, 24 or 72 h Exposure of Human Lung-Derived Cells to $KNbO_3$, $LiNbO_3$, $BaTiO_3$, KTP, and ZnO Nanoparticles (50 μg/mL)[a]**

	% of surviving cells				
5 h	$KNbO_3$	$LiNbO_3$	$BaTiO_3$	KTP	ZnO
A549	81.2 ± 4.4	78.6 ± 3.7	91.0 ± 5.3	76.3 ± 4.2	57.7 ± 7.8
HTB-182	81.6 ± 6.7	82.6 ± 2.7	87.1 ± 9.4	67.8 ± 2.3	41.6 ± 7.8
HTB-178	74.3 ± 2.1	90.7 ± 8.8	87.7 ± 12.7	78.4 ± 6.2	27.8 ± 6.7
BEAS-2B	84.9 ± 5.3	87.9 ± 7.1	93.1 ± 0.6	63.2 ± 5.6	29.9 ± 6.7
24 h	**$KNbO_3$**	**$LiNbO_3$**	**$BaTiO_3$**	**KTP**	**ZnO**
A549	82.0 ± 3.6	84.7 ± 5.7	84.7 ± 3.8	67.8 ± 2.5	17.7 ± 5.3
HTB-182	73.7 ± 6.7	80.9 ± 3.5	85.5 ± 1.3	61.4 ± 6.0	4.5 ± 1.0
HTB-178	74.2 ± 1.0	83.6 ± 3.3	91.8 ± 1.7	76.8 ± 7.7	6.8 ± 1.2
BEAS-2B	81.7 ± 4.0	93.9 ± 4.9	92.1 ± 3.7	55.8 ± 5.3	9.9 ± 3.3
72 h	**$KNbO_3$**	**$LiNbO_3$**	**$BaTiO_3$**	**KTP**	**ZnO**
A549	69.8 ± 4.1	73.5 ± 5.2	81.4 ± 8.2	NA	NA
HTB-182	77.4 ± 4.5	80.5 ± 5.5	88.9 ± 2.3	NA	NA
HTB-178	56.9 ± 5.7	81.9 ± 6.5	74.5 ± 9.1	NA	NA
BEAS-2B	64.4 ± 4.9	70.9 ± 4.3	78.2 ± 9.1	NA	NA

[a] A549, HTB-182, and HTB-178: human lung cancer cells; BEAS-2B: nontumoral lung-derived cells. Results are the mean ± SD of triplicates of two independent experiments.



The main results (reported on the tables TP3.T1) have been published in the high impact journal in the field:

Table TP3.1. TP3 SHG Nanomaterials properties: TABLE 1, hyperpolarizability properties, TBLE 2, Cytotoxicity effect after 24h and 72 h exposure.
Figure (a) Room temperature magnetic hysteresis loops of BFO sample BFO-LT2. (b) Magnetic separation of BFO particles in water suspension. (*Extracted from Harmonic Nanocrystals for Biolabeling: A Survey of Optical Properties and Biocompatibility, D. Staedler, T. Magouroux, R. Hadji, C. Joulaud, J. Extermann, S. Schwung, S. Passemard, C. Kasparian, G. Clarke, M. Gerrmann, R. Le Dantec, Y. Mugnier, D. Rytz, D. Ciepielewski, C. Galez, S. Gerber-Lemaire, L. Juillerat-Jeanneret, L. Bonacina, and J.-P. Wolf, ACS Nano, **6** (3), 2542–2549 (2012) [DOI: 10.1021/nn204990n)*

Identification of a nanomaterial combining magnetic response, high nonlinear optical efficiency, low cytotoxicity: Bismuth Ferrite (BFO). Optimization of its cost-effective synthesis in large volume production

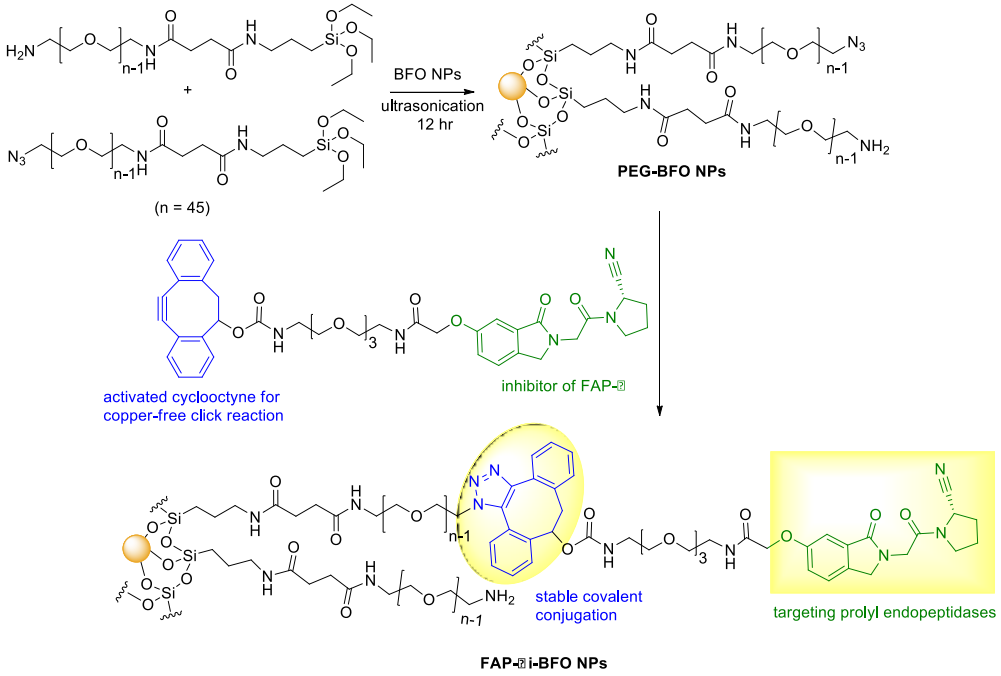
In parallel to the work described in the above ACS *Nano* paper, the partners have been also developing bismuth ferrite nanoparticles for a wide range of applications. The partners took an advantage of the combustion method for the direct formation of a crystalline precursor of BFO following the first reaction step. In respect of the standard sol-gel reactions (previously reported in the scientific literature and always used for non-biological applications), lower annealing temperatures can be foreseen to obtain phase pure BFO, preventing Ostwald ripening and resulting in powder with better crystallinity and smaller crystallites. The BFO nanoparticles obtained following this approach have been thoroughly analysed by the partners. The samples obtained thereby outperform all other HNPs candidate nanocrystals as they display normalized hyperpolarizability values (<*d*>) of the order of 80 pm/V (as compared to <18 pm/V of other materials) and also a clear magnetic response that can be used for nanoparticles attraction and possibly magnetic detection. For this last application, however, a further increase of the magnetic response would be important. FEE and UdS continue to work towards the synthetises of core-shell structures based on BFO and iron oxide with enhanced magnetization efficiency.

Concerning the nanomaterial biocompatibility, experiments were designed and performed to establish the effects of BFO NPs in cell metabolism and the uptake mechanism of these NPs. While bare BFO NPs formed

aggregates in biological relevant media, particularly when the media were supplemented with serum, and induced weak haemolytic effect on human red blood cells (HRBc), PEG-BFO NPs were significantly less prone to aggregation and displayed almost no hemolytic effect on HRBc. These data confirmed that the protocols developed at EPFL for the PEGylation of BFO NPs improved their colloidal stability and biocompatibility. In addition, the partners demonstrated that PEG-BFO NPs, in concentration range of 1-10 µg/ml, did not show any significant toxicity in cell models and were internalized in intracellular organelles, in particular in the lysosomes, remaining located into the cells for several days.

Synthesis of targeting ligands for several cancer cells biomarkers and validation of their affinity for the selected biomarkers. Conjugation of the targeting ligands to nanoparticles

Based on the successful protocols developed at EPFL for the coating and functionalization of Fe_3O_4 with multifunctional ligands targeting αvβ3 integrin, HER1/HER2 or fibroblast activation protein α (FAP-γ), the EPFL partner concentrated their efforts on the synthesis of homogeneous and highly pure samples of BFO NPs targeting prolylendopeptidases expressed at the surface of cancer cells and in tumour environment. A method based on copper-free click reaction allowed for the efficient functionalization of PEG-coated BFO NPs, using simple washings of the suspended NPs with organic solvent to remove unreacted ligand (see scheme TP3.1). This procedure avoids: (i) complex separation and purification steps, (ii) contamination of functionalized BFO NPs with metal catalytic species. The resulting samples have been made fully suitable for subsequent evaluation in human living cells and tissue samples.



Scheme TP3.1. Preparation of functionalized BFO NPs

Labelling of cancer cells and imaging through multiphoton microscopy

The first stage of biological evaluation studies addressed the ability of FAP- αi-BFO NPs to selectively label cancer cells and the opportunities for imaging of the labelled cells by multiphoton microscopy. Human lung-derived NCI-H520 cancer cells and BEAS-2B non-cancerous cells were exposed to PEG-BFO NPs or functionalized FAP-αi-BFO NPs, subsequently labelled with fluorescent cell membrane probes and NPs were further revealed by detecting their SHG signal. In addition, a competition assay using pre-incubation of the cells with the non-conjugated inhibitor of FAP-α, has been implemented to address the target specificity of the probe binding (Figure TP3.1).

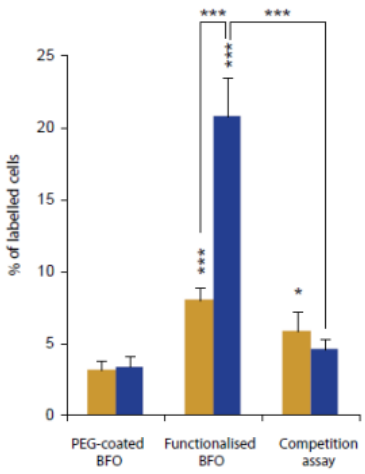


Figure TP3.1.Target-specificity assay: comparison in labelling efficiency between **PEG-BFO NPs**, functionalized **FAP-ai-BFO NPs** and competition assay (using pre-incubation of cells with the non-conjugated inhibitor of FAP-a).

Both cancer and non-malignant cells were reliably labelled with **FAP-αi-BFO NPs**, but the number of cancer cells associated with **FAP-αi-BFO NPs** was significantly higher than for non-tumoral cells (see histogram). Moreover, in the competition assay the quantification of the association clearly revealed that the binding was target-specific. These results suggest that BFO NPs can be used as labelling nanoprobes targeting proteins expressed at the surface of cancer cells. However, the selectivity factor between tumoral and non-cancer cells needs to be further optimised.

Assessment of the imaging and detection potential of HNPs for wider applications and development of new imaging modalities based on the extension of the infrared multi-photon technology

An essential input of the TP3 in NAMDIATREAM was the development of nonlinear imaging modalities for nanoparticles-based cancer diagnostics. In this respect, the development of a new microscopy system enabling the exploration of longer wavelengths (up to 1.3 µm) and providing the possibility of handling large samples by Nikon partner has been of a paramount importance. In collaboration with Max Plank Institute (Gottingen) and the Institute Curie in Paris (Nikon imaging centre in France,) tissues from xenograft murine tumours labelled by functionalized BFO HNPs were imaged at different wavelengths. The results obtained indicate that even with very thick tissue samples, second harmonic emission of HNPs can be easily detected with excellent contrast against the endogenous background (mostly imposed by collagen). Even more importantly, these measurements allowed the detection of third harmonic emission from the nanoparticles, a result not foreseen previously and deserving further fundamental investigations. Notably, the correlation among second and third harmonic signals emitted by HNPs and excited simultaneously by the same infrared laser source can be used to strongly increase the selectivity of the technique, as reported in the image comparison figure below. This finding establishes a novel multi-harmonic coherent imaging modality and a relevant high profile scientific manuscript has been submitted (Figure TP3.2).

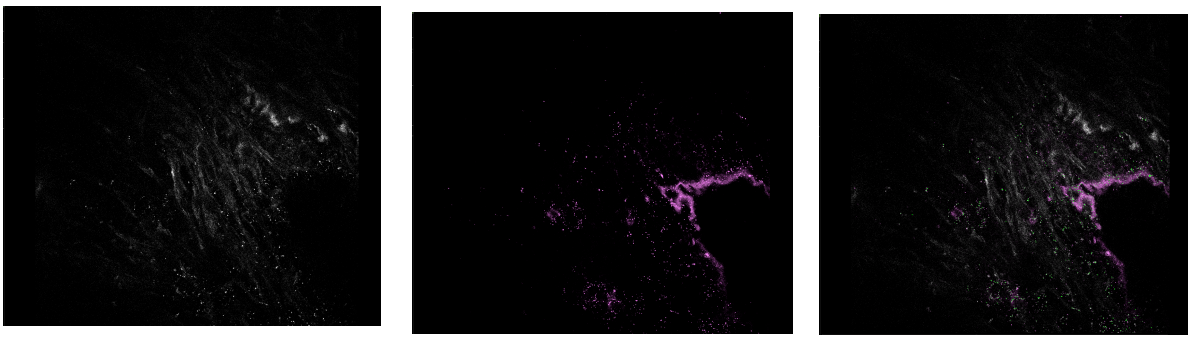


Figure TP3.2. From left to right: second harmonic (SH), third harmonic (TH), and SH+TH correlation image of a xenograft tumor model labelled by HNPs. The correlation signals (in green) identify with high selectivity the HNPs against the background by collagen (SH) and lipids (TH).

From left to right: second harmonic (SH), third harmonic (TH), and SH+TH correlation image of a xenograft tumor model labelled by HNPs. The correlation signals (in green) identify with high selectivity the HNPs against the background by collagen (SH) and lipids (TH)

A similar approach had been previously tested by UNIGE in a flow detection assay based on the detection of individual nanoparticles in plasma by simultaneous second and third generation harmonic emission. The results published in the *Nanoletters* journal attracted the attention of the BD industrial partner, and the

company decided to test the approach in a commercially validated system. As a result, BD with UNIGE have further developed the concept, which now has a relevant commercial application in the field. This advance is expected to be further pursued by the partners beyond the lifespan of NAMDIATREAM, provided that targeted funding is secured.

Over the duration of the project, complementary applications of HNPs enabling to assist in generation of stem cell derived tissues (for prospective medical applications, like, for instance, tissue regeneration healing after myocardial infarction) were offered, in collaboration with TCD. In particular, the high speed scanning capability of the Nikon multiphoton microscope was utilised to reconstruct, in 3D real time, the contraction of stem cells (derived from tissue). This allowed the recording of critical functional response parameters (e.g., contraction period, spatial and temporal organization of individual cell movements).

Proof-of-principle demonstration of wavelength-selective photo-interaction with cellular DNA for theranostics applications

Most recently, TP3 partners have proposed and demonstrated an approach of direct photo-interaction by HNPs and nuclear DNA. This could be used for light triggered activation of molecules in a tissue (i.e. uncaging of cancer drugs attached at the surface of nanoparticles); as presented in the latest *NANOSCALE* publication where a method decoupling imaging and therapeutic intervention has been described. This is reached by selecting each modality by switching the excitation laser wavelength from infrared to visible. In particular, the generation of ultraviolet radiation (wavelength = 270 nm) allows direct interaction with DNA in the absence of photosensitizing molecules. The approach proposed reduces side effects and diffusion of toxic compounds, increasing selectivity and localization of treatment.

Expected industrial exploitation outcomes of TP3 include:

- New approaches enabling **in depth imaging** of healthy and cancer tissues.
- Utilisation of non-centrosymmetric nanoparticles developed and validated within the project.
- **Licensing and commercialisation:** currently three nanoproducts based on non-centrosymmetric nanomaterials are **licensed and commercialised** to TIBIO Sagl.

TP4: Magnetic nanowire Barcodes for disease marker detection (MagBar), TP Leader – TCD

TP4 was industry-driven by key small and medium enterprises (SME) and BD Bioscience France multinational company partners to develop and test the functionality of the magnetic barcode prototype system for detection of disease biomarker molecules in the biological fluids and on the surface of living cells.

TP4 team effort over the four years of the project has been focused on the development and validation of sub-micrometre bench-type flow cytometry system for cancer biomarker detection using nanoprobes in the form of nanoparticles and nanowires. Flow cytometry design enables to register micrometre size objects (such as cells and micro-particles). Enhancement of its detection capabilities towards the nanometre range was one of the objectives of this project, especially in application to such unconventional objects as nanowires. Under the leading guidance of BD, scientific and industrial expertise, and with the contribution of CLX and RDS innovative technological solutions based on microfluidics and high throughput optical detection system respectively, a bench-top MagBar device has been developed, Figure TP4.1.

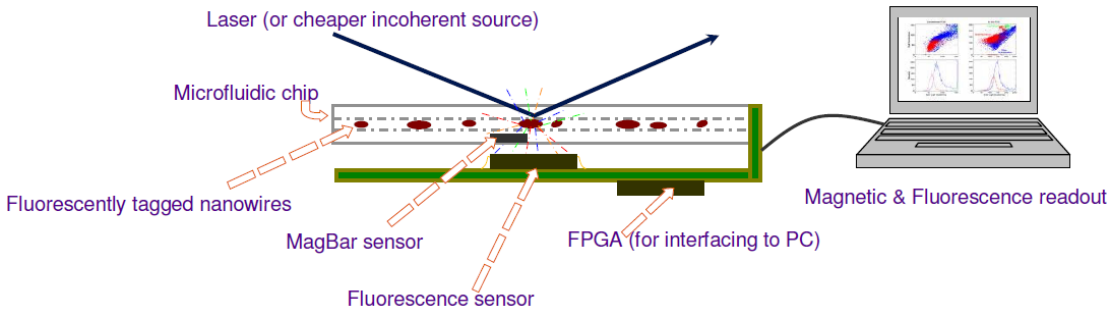


Figure TP4.1. Schematic of the integrated MagBar technology platform prototype

TP4 research and technology development work within NAMDIATREAM has been centred on the three main areas:

i) manufacturing of lab-on-a-wire probes,

ii) magneto-optical detection of lab-on-a-wire probes (MagBar system),

iii) detection of clinically validated soluble markers for breast, lung and prostate cancer.

Manufacturing of lab-on-a-wire probes

The fabrication of magnetic probes with shape anisotropy such as nanowires, (including barcoded magnetic nanowires) implementing template electrodeposition posed several challenges that have been successfully overcome through the joint work of TCD-PHYS and TCD-CHEM. The nanowires produced have been validated both for flow cytometry and the MagBar device application (described below).

Within the first 18 months of the project, the delivery of monodispersed magnetic nanowire samples as single or varying segment length was provided to the project as part of Work package 2 (D2.8). The fabrication and characterization of multi-segmented nanowires by electrodeposition techniques allowed the supply of magnetically detectable nanowires ("lab-on-a-wire") to be used as barcodes for identification of clinical cancer markers, Figure TP4.2. Spherical nanoparticles were also used as calibrator controls for sensor detection and assay validation (D4.10). Nanowires and nanoparticles had to meet high standards of colloidal stability in aqueous-based solution for diagnostic assay. (D2.9).

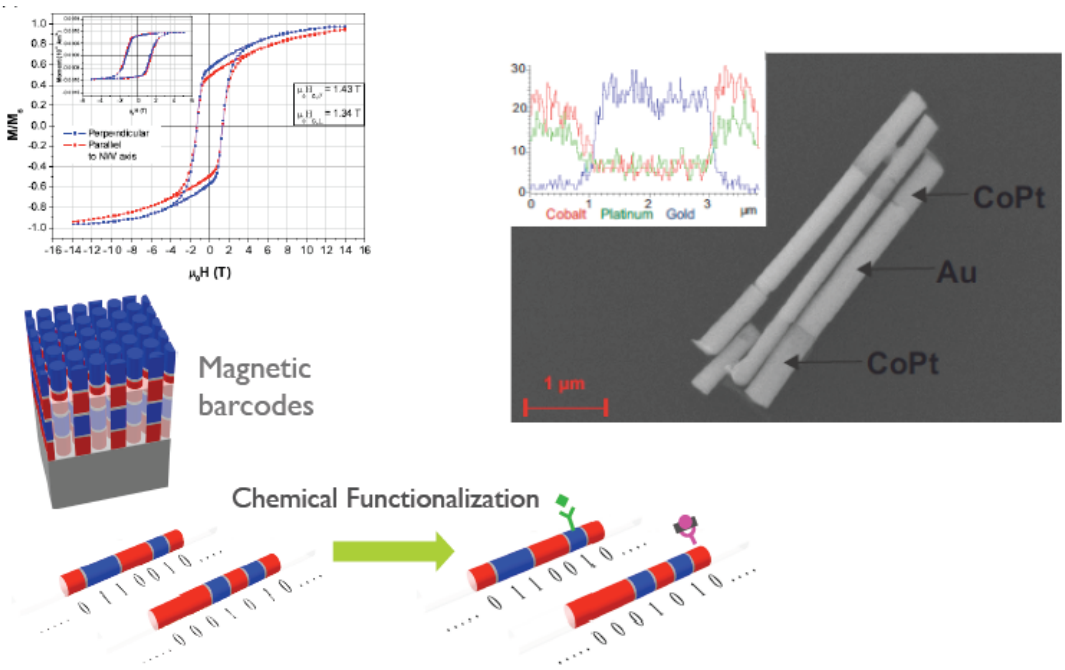


Figure TP4.2. Magnetic Barcode Nanowires: magnetic characterization, properties and SEM imaging.

By month 36, probes surface functionalization has been carried out by TP4 partners through click chemistry whereby covalently functionalised biochemical moieties were put onto the nanowire surface in order to achieve a reliable immunofunctionalised probe. Herceptin (clinical gold standard), fluorescently labelled ErB2, EGFR and PSA markers were the chosen as markers. Screening of the most efficient commercial markers was also undertaken in order to achieve the highest efficiency for the diagnostic assay. Characterization of the marker binding strength was assessed by liquid Atomic Force Microscopy, fluorescence labelling onto nanoprobes was assessed by the Laser Confocal Microscopy Imaging. Both techniques provided qualitative and quantitative information on the probes characteristics. Dynamic characterisation by flow cytometry was subsequently carried out for comparison between nanoprobes and standard calibrator beads, to assess different assay efficiencies and to compare with the optical sensor platforms developed by RDS for the MagBar systems, Figure TP4.3.

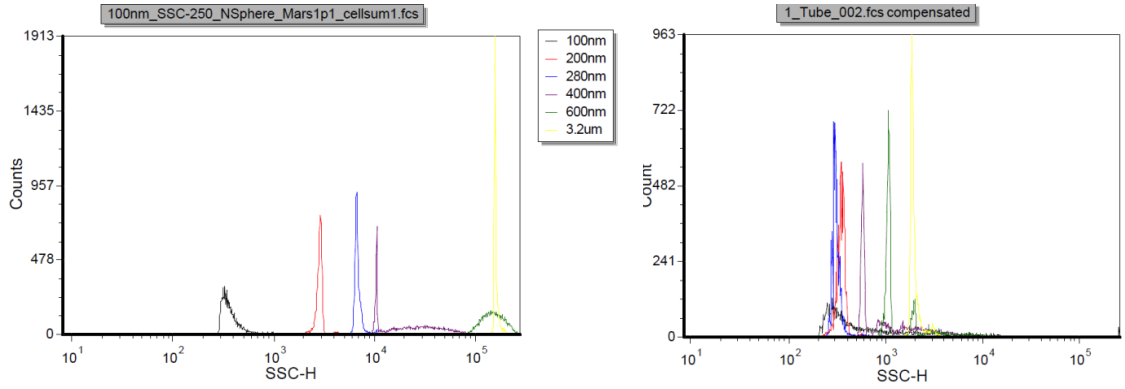


Figure TP4.3. Comparison of detection of nanoparticle ladder between Radisens Diagnostics (Left) and BD FACS CantoA (right). Fluorescent nanoparticles used ranged from 100 nm to 3.2 micrometre.

MagBar system: over the initial 30 Months of the project, proof of concepts of "lab on a wire" magneto-fluorescent sensor system and magneto-fluorescent probes modelled, designed and developed within work packages 1 and 4 have been characterised, tested and benchmarked against calibrators. As a result, integrated system was developed and assembled by miniaturisation and optimisation of Giant Magneto Resistance sensor (TCD-PHYS) and 4 Colour Sensor Module (RDS), initially in a lab based test rig (D4.7, D4.8) and then integrated together within a bench-top platform (D4.10, and D5.5). Validation was subsequently carried out for each sensing and detection device by calibrator materials such as magnetic micro- and nano particles (D4.10, D5.5).

Towards Month 36, CLX partner has primarily worked on re-engineering existing microfluidic platform integrating magnetic sensors and optical system. The setup for single channel magnetic barcode experiment was based on Cellix's VenaFlux Platform^{TM} adapted for hydrodynamic focused flow for magnetic sensing. Integration of the RDS optical detection technology under BD technical specification allowed to improve the throughput of magnetic nanowire detection and to transform the system into "**flow cytometry -like setup**" (D4.9). The integrated demonstration platform prepared is composed of the following units, (D6.4):

- Epi-fluorescence detection system (EDS) from RDS,
- Data acquisition system (DAS) from RDS partner,
- Capture Software from RDS partner,
- Flow cell for focused flow from CLX,
- Biochip carrier and carrier frame from CLX, GMR sensor plate and front-end electronics from the TCD-PHYS,
- Sheath and sample pumps from CLX.

The demonstrator platform (industrially developed) was completed in April 2014 and has been tested since. Three identical platforms are setup for testing at CLX, RDS and TCD-PHYS; prototype in Figure TP4.4.

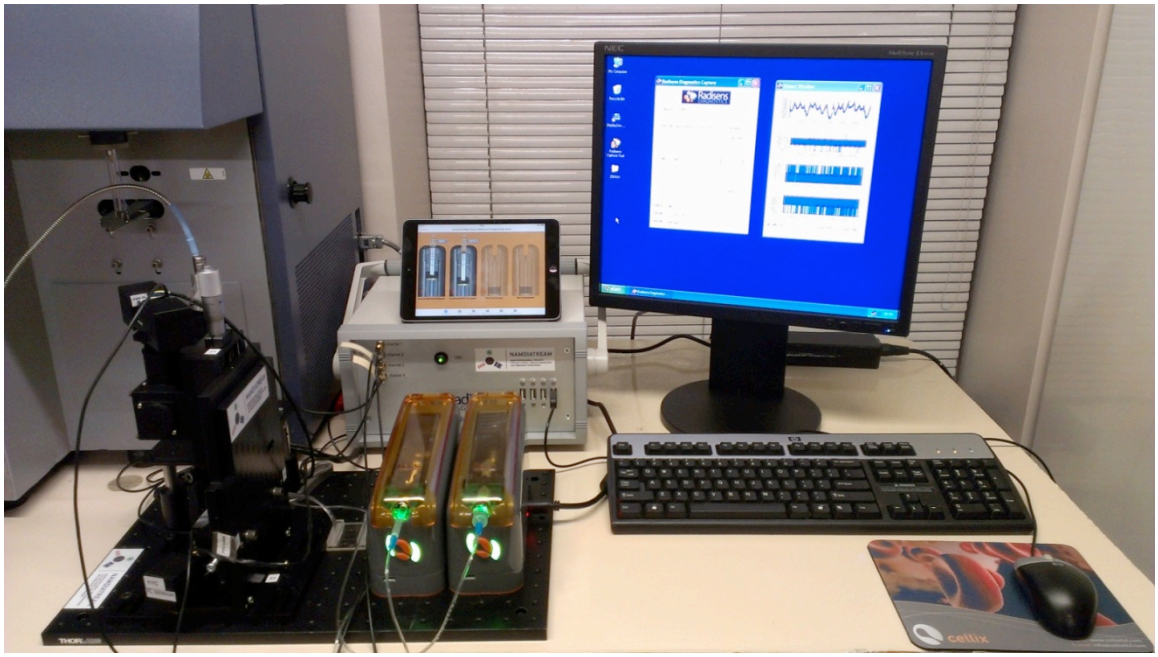


Figure TP4.4. Integrated Mag-Bar demonstrator platform technology

The demonstrator prototype enables the multiplexed detection of molecular biomarkers of human diseases in a high-sensitivity and throughput format using magnetic nanowire has the major advantages over existing and developing methods:

i) it requires a cost effective low power laser for optical detection and confirmation,

ii) it is not using any complex biochemical preparation or diagnostic procedures,

iii) low volumes of samples and reagents are needed,

iv) it uses miniaturised fluorescent sensors with multiple integrated narrowband filters at very low cost and complexity with cytometric detection,

v) high-throughput capabilities are offered.

The successful implementation of MagBar prototype by TP4 has developed a new concept in the development of nanoscale flow cytometry for the detection of cancer biomarkers. This enabled both CLX and RDS to develop new commercial products, which have been, or expected to be, launched within 2015.

Detection of clinically validated soluble cancer markers for breast, lung and prostate cancer

Over the first half of the project life span, TCD-MED work progressed towards the preparation of customised magnetic and fluorescent nanoprobes for the detection of breast, lung and prostate cancers optimised for the chosen cancer biomarkers (Herceptin, ErbB2/HER2, EGFR and PSA) and cell models. Proteins obtained from cell lysates and subject to Western blot analysis were quantified and compared to the respective soluble biomarkers present in spiked samples provided by PGK partner as part of TPH activity (see below). Ranking between cell models and clinical samples has been achieved as part of the Work Package 3, 5 and 8 activities and enabled further advanced work with nanotechnological probes (e.g., quantum dot particles and magnetic nanowires).

Over the last 24 Months of the project, progress towards the detection of human soluble markers has been pursued by using the suite of markers (stated above) with flow cytometry (clinical gold standard analytical

tool) and with the MagBar system. Detection of recombinant human soluble markers in blood plasma was carried out using spherical and nanowire functionalised probes under different experimental conditions and benchmarked against the gold standards. Laser Scanning Confocal Microscopy imaging allowed for assay performance assessment and cell-specific marker expression evaluation.

Detection of cancer biomarkers (ErbB2, EGFR & PSA), using MagBar platform has been carried out by TCD-Med in cooperation with PGK and BD. Assay procedures for detection of biomarkers in simple single analyte systems were developed by TCD-MED, PGK and BD, optimised to be utilised by flow cytometry with the MagBar system as shown in Figure TP4.5.

High impact factor publications have been produced as a result of this work.

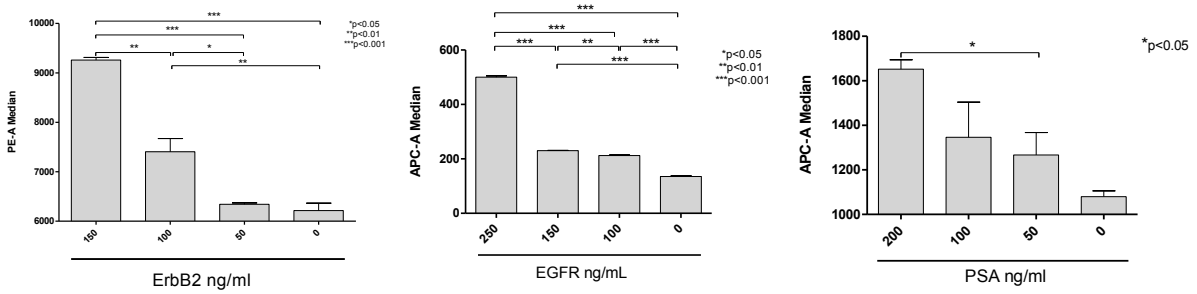


Figure TP4.5. Detection of cancer biomarkers in single analyte systems using nanowire platform. Immunofunctionalised nanowire platforms were used to detect purified biomarkers in single analyte systems in a proof-of-principle study. Titration of the purified proteins sErbB2, EGFR and PSA allowed the concentration-dependent response of the system to be assessed. In each case, when compared to bovine serum albumin (BSA), the nanowire platform was found to be specific for the intended target.

In summary, the foreseen industrial outcomes of TP4 include:

- **New concept** in the development **of nanoscale flow cytometry** has been established,
- **Optical-magnetic fingerprinting** of non-conventional nanoprobes **has been achieved**,
- New **technology platforms** based on MagBar have been **transferred to SME partners**.

TP-Horizontal – TP leaders –PUM, UCD / TCD, MPG

The evaluation of the colloidal stability of functionalised nanoprobes (NPs) in biological media, their applicability in tumour models and subsequently their environmental toxicity has been part of the horizontal activities across all the TPs of the project.

These activities are reflected in several tasks, which are parts of the Work packages 3 and 7; whereas Work package 8 was focused to the validation of the nanoprobes regarding their specificity and sensitivity using biological samples obtained murine and human samples (under approved ethical licenses.)

TCD, with the contribution from the other TPH consortium partners, developed and applied a cross-disciplinary, three-tiered approach for high throughput assessment of nanoprobes safety. This allowed to establish a baseline data for evaluating the potential risk associated and toxicity of the different nanotechnological probes. Physico-chemical characteristics, safety profile of the components embedded in the devices and technological platforms were subjected to robust assessment. The methodological approach implemented a systematic high throughput testing of bulk ("ex-synthesis" produced) nanoprobes, while more refined investigations were carried out on the functionalised nanoproducts (Figure TPH.1).

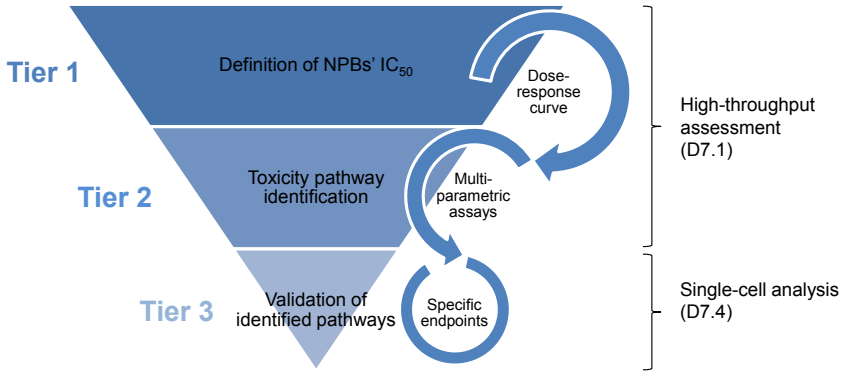


Figure TPH.1. Schematic of the three-tiered, high throughput approach used in Work package 7 to define a key set of baseline data for evaluating the potential toxicity of the starting NPs embedded in the devices and platforms being developed in the project.

Similar approach within the Work package 7 has been adopted across the other tasks of particular importance for the risk assessment of the device components and the assembled devices (D7.8), which applied to all the technology platforms. Only the NPs, which were accepted as stable with shelf life of weeks, were further screened for safety. Four key NPs were selected, stabilised by PUM (under Work package 2), and utilised by all the TPs.

A three-tiers toxicity screening was developed for the assessment of chosen NPs. Main objective was the identification of a dose-response relationships for *in vitro* tests (Tier 1) and on evaluating perturbations in toxicity pathways expected to lead to adverse health outcomes if the perturbations were sustained *in vivo* at a sufficient level of intensity and duration of exposure (Tier 2 and 3). The high-throughput results of this specific work, belonging to Tier 3 (associated with single-cell analysis, as for Figure TPH.2) resulted in the "go" / "no go" safety-decision-making-tool for the probes to be utilised as diagnostic probes. Regulatory guidelines and reflection papers were also considered for potential industrialization of the nanoprobes, in conjunction with the results achieved under Work package 7. High impact factor publications were achieved as part of this work.

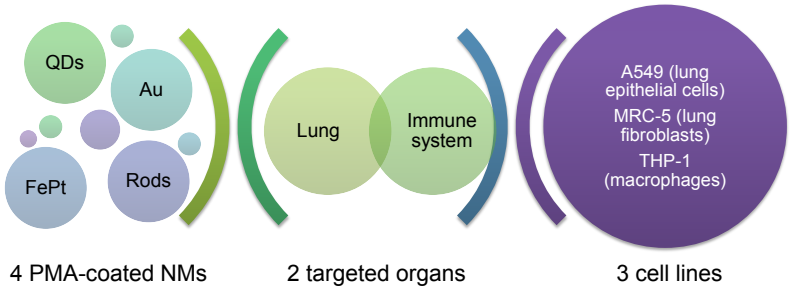


Figure TPH.2. Summary of NPs and cell lines tested, as well as targeted organs, for the high-throughput multi-parametric safety assessment of NPs

Further to that, the validation of the quality of the nanoprobes produced from the different TPs was assessed in small animal models. Particular focus was given to the QDs probes developed within the TP1 with a direct utilization as diagnostic probes for histopathology applications. Therefore, the assessment of the specificity and sensitivity using biological samples obtained from tumour bearing mice was of a prime importance. The nanoprobes tested under this task were composed of highly fluorescent quantum-dots (QDs) conjugated with the antibodies recognising specific tumour markers, i.e. single domain antibodies against HER2, EGFR and CEA (HER2-QDs, EGFR-QDs and CEA-QDs). Using metastatic human tumour mouse models the validation of the nanoprobes was performed by biological assays. From these mouse models,

bone marrow, blood samples, various organs and the tumour have been collected and were investigated for the occurrence of disseminated HER2, EGFR or CEA positive tumour cells/circulating tumour cells (DTC/CTC) by applying nanoprobe technology. Human metastatic models were established, adopted for the validation of TP1 probes for immunostaining. By immunostaining of sections of organ tissues, it was possible to detect HER2 specific positive disseminated human tumour cells in 2,5 µm paraffin sections and in 50 µm agarose sections of different organs including brain, testis, lung, liver and lymph nodes (Figure TPH.3).

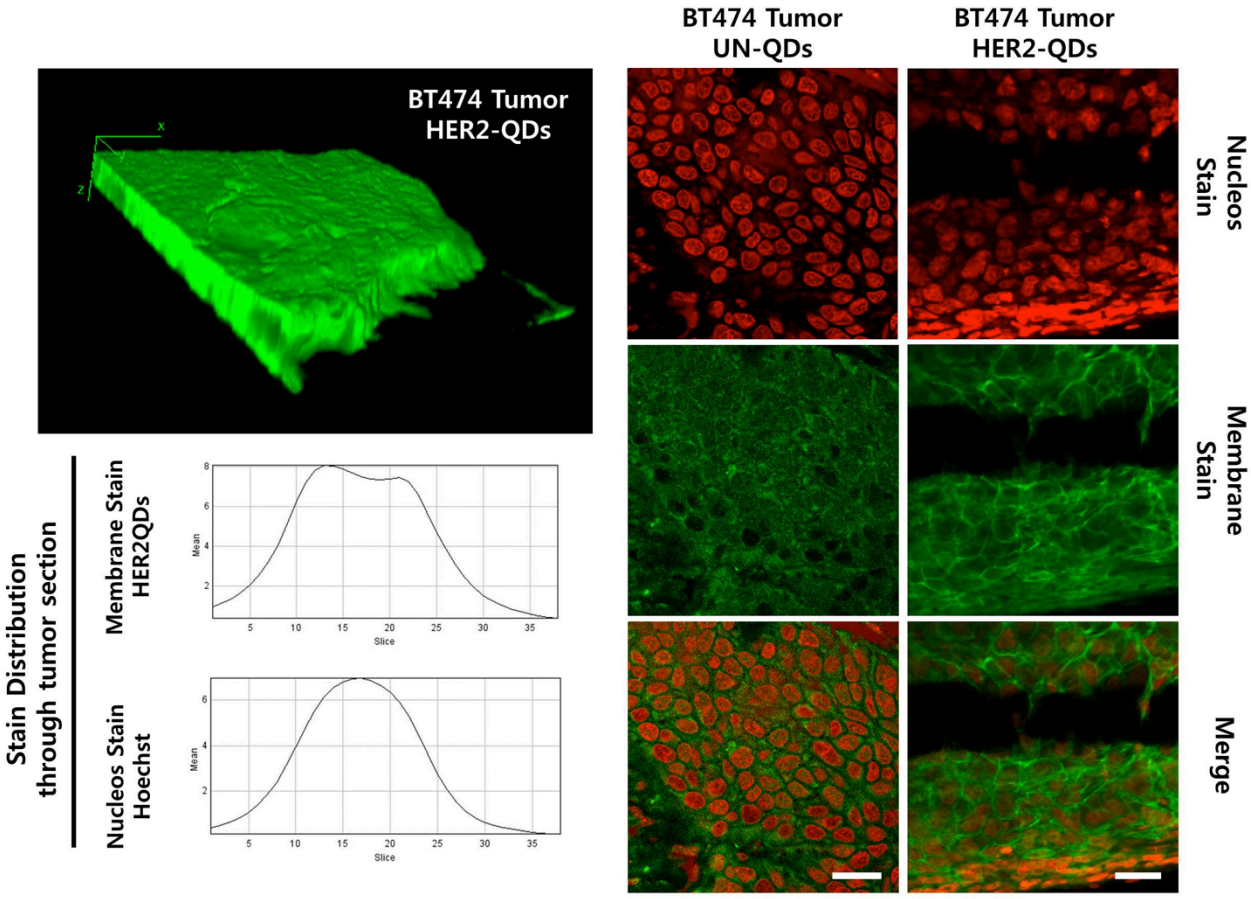


Figure TPH.3. Detection of HER2 protein using two-photon microscopy in breast tumour sections of BT474 xenograft mice. The two-photon images show the stain for HER2 protein (green) in a 50 mm breast tumour section using anti HER2-QDs nanoprobes. Hoechst (red) was used to stain the nucleus. On the left side the graphic show the distribution of the signal through the 50 mm breast tumour section. The wavelength excitation used was 850 nm. Scale bar = 20 mm, x = y = 250 mm and z = 50 mm.

Validation of the industrial demonstration prototypes developed in the Work package 6 involved large series of real samples from different sources (blood, saliva, urine and biopsies). Furthermore, the development of the multiplexed bead-based immunoassays in Luminex technology, and technical validation of such assays was also performed, in order to ensure that the quality of the samples would meet the reproducibility and reliability standards imposed. Different technical acceptance parameters were established based on normalized guides, such as FDA guides for *in-vitro* immunoassays.

The summary of such clinical validations of the immunoassays performed with the Luminex technology is given in Table TPH.1.

Immunoassay (biological sample)	Marker	Univariate Analysis	Multivariate Analysis
4-plex Gynaecological cancer (Serum)	Her2	> 0.05 (ns)	> 0.05 (ns)
	CEACAM	> 0.05 <0.1 (borderline)	
	EGFR	> 0.05 (ns)	
	EpCAM	> 0.05 (ns)	
3-plex lung cancer (Serum)	LCM1	< 0.05 (sig)	< 0.05 (sig)
	LCM2	< 0.05 (sig)	
	LCM3	< 0.05 (sig)	
3-plex lung cancer (BAL)	LCM1	< 0.05 (sig)	< 0.05 (sig)
	LCM2	< 0.05 (sig)	
	LCM3	< 0.05 (sig)	
3-plex prostate cancer (Urine)	PCM1	< 0.05 (sig)	< 0.05 (sig)
	PCM2	> 0.05 (ns)	
	PCM3	> 0.05 (ns)	
1-plex prostate cancer (Urine)	PSA	< 0.05 (sig)	

Table TPH.1. Summary of the statistical results of the clinical validations of all immunoassays performed with Luminex .

Finally, expected outcomes of TPH include:

The horizontal activities have been focused on securing groundbreaking progress in these three main areas:

- overall **quality assurance on the nanoprobes** preparation, characterization, colloidal stability, and water dispersion (pre-functional cancer labelling)
- **assessment of standardisation, safety and risks across all the technological platforms**
- development of suitable representative models for **nanoprobes** testing towards their **preclinical validation**.
-

Potential Impact

Economic impact

Despite a strong progress in medical research, cancer still remains one of the most frequent causes of death worldwide, and over 1.7 Million cancer-related deaths are registered in Europe every year. Although some types of cancer can be treated more successfully than the others, it is well known that one of the most effective ways to improve cancer treatment is by improving its early diagnosis. NAMDIATREAM project addressed this pressing social need through the development of ultra-sensitive devices for the early diagnosis of three most widespread malignancies, such as lung, breast and prostate cancer. The consortium has developed **non-invasive, easy to use *in vitro* diagnostic devices featuring outstanding sensitivity and specificity,** which can be safely implemented for the ultimate benefit of the patients and stakeholders.

Through the engagement of four complementary Technology Platforms, NAMDIATREAM has developed highly sensitive "lab-on-a-bead", "lab-on-a-chip" and "lab-on-a-wire" nanotools enabling quantitative detection of cancer markers on the surface of malignant cells and in biological fluids at the early stages of cancer development.

The high sensitivity and specificity of the nanotools developed by the NAMDIATREAM project offer a qualitatively new approach to cancer diagnostics in micro-litre volumes of biological fluids (blood, urine, saliva), minimising the invasiveness of the procedure to the patients and reducing the costs of the clinical tests. This fosters the early detection of cancer by means of routine population screening, facilitates prompt intervention to treat the disease and permits a more effective evaluation of the disease progression following treatment paving the way to the accomplishment of the consortium strategic goals:

- to reduce cancer-associated mortality and societal costs for national healthcare systems,
- to increase the quality and expectancy of life of citizens in Europe and worldwide.

NAMDIATREAM SMEs and Multinationals (MNCs) involvement within the project has been focused in securing technological breakthroughs contributing to Europe's competitiveness in diagnostic and imaging products, in line with the forecasted **€** 50bn global market by 2018 (Frost and Sullivan report) in In Vitro Diagnostics (IVD). With revenues of **€**35bn (2012), Western Europe is the second largest IVD market in the world, after the US, growing at 11.4% CAGR to **€**12.6bn (2012) (source: IVD MarkeTreach Enterprise Analysis, 2012).

The global IVD market is geared up for considerable and steadily increasing future growth as the healthcare industry is becoming more focused on predictable patient treatment and outcomes, targeted medicine, earlier disease detection, screening and hi-tech automation. Technological advancement of IVD is bound to contribute to significant healthcare cost savings.

NAMDIATREAM has identified, through an industrially driven exploitation plan, that the project outcomes will target the diagnostics laboratory practice (by offering clinical and research diagnostic kits, biochips, functional nanoprobes and reagents), instrumentation and medical devices, and point-of-care diagnostics.

NAMDIATREAM exploitable outcomes achieved by the end of the project (July 2014), have enabled the industrial partners (both MNCs and SMEs) to gain prominent insight knowledge and commercial advantages in the dedicated IVD segments such as molecular diagnostics, immunoassays and point of care device development associated with potential high revenues and mid to high forecasted growth rates (see Figure IMP2.1).

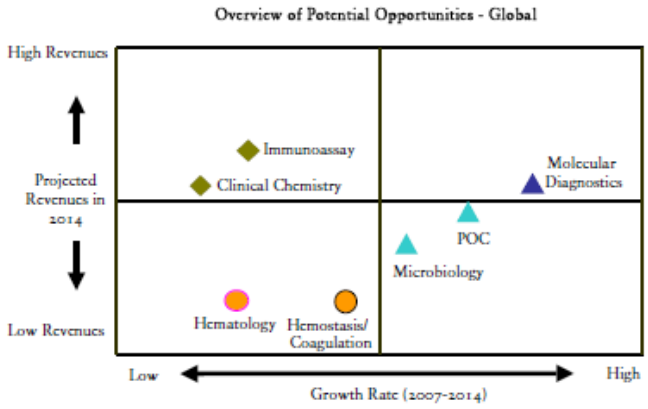


Figure IMP2.1: Overview of potential opportunities in the Diagnostic market

With such substantial deliverables and potential opportunities on the horizon alike, NAMDIATREAM project has successfully promoted several innovations in the field of early cancer diagnostics. The project has achieved important new benchmarks applicable for further scientific research, valuable Know-Hows for commercial usage and clinical practice.

As described above in the scientific and technical section, the main innovations achieved by NAMDIATREAM can be summarised as follows:

1) Q-Dots: In general, the nanoparticles of this type have a very long fluorescent emission lifetime and can effectively compete with any existing organic fluorochromes currently used for biomedical applications. Their unique physicochemical properties make them applicable in a much wider field of science, as they can scatter light and transform light due to their semi-conductive nature. In addition, our ultrasmall Qdots-based probes will be of a particular value for labelling intracellular structures and/or membrane receptors,

2) Single-domain antibodies have been only recently introduced into the biomedical sciences and when matched with engineered nanomaterials such as nanoparticles or nanowires, provide new approaches to developing of ultrasmall immunoprobes. The perfection of labelling procedures and the reliable performance of them holds a real competitive advantage against the currently available diagnostic tools,

3) Lab-on-a-wire techniques hold the promise of providing a fingerprint of proteins of interest directly from the biological fluids. Other and competing techniques are available and industrial benchmarking will be required to understand the true potential of these unconventional probes utilised for IVD assays,

4) The PlasMag technology has been developed and proven as a fast and simple “mix-and-measure” type in-vitro diagnostic method to specifically quantify protein biomarkers directly from unprocessed liquid samples. It has the potential to provide a universally applicable point-of-care platform for the fast and improved clinical decisions,

5) The Second Harmonic Generation technique holds the promise of noiseless signal generation and thus the delivery of an enhanced sensitivity. This technique enables the single cell identification, analysis of new drugs’ distribution, uptake and metabolism in deep tissue locations,

6) The MagBar technology presents an attractive technique for the evaluation of the protein biomarker characteristics (natural size, configuration, interactions) based on the unique flow cytometry profiles recorded within the lab-on-a-wire probe. The technique would be an ideal research tool for subsequent laboratory screening approaches.

NAMDIATREAM industrial partners’ expertise has facilitated a comprehensive market analysis, which led to the defining of the project exploitation strategy on the above listed innovating technologies. **One of the measureable outcomes of such effort was the licensing of one of NAMDIATREAM generated IP to TIBIO Sagl, Comano (Switzerland) now commercialising three of NAMDIATREAM nano-products.** Further

industrial translation is foreseen onwards from the 2015, which will have a sustained impact on the European IVD market and will subsequently affect the Europe's global competitiveness in this area.

Social impact

NAMDIATREAM diagnostics toolkits are designed to target three of the most frequent cancer types: lung, breast and prostate.

- **Lung cancer** *is the leading cause of cancer death in the world. In Europe (2012) it caused* ***353,000 deaths*** *(254,000 men, 99,000 women). Values which are even expected to grow in the future since in 2013, 409,000 new cases of lung cancer (290,000 men, 119,000 women) were diagnosed.*

  The prognosis for lung cancer is poor, with 5-year survival rates being less than 12%. This is explained by the fact that most patients have metastases at the time of diagnosis. Thus, effective tools to diagnose lung cancer at an early stage can dramatically reduce lung cancer mortality.

- **Breast Cancer** *is the major cancer affecting women. In Europe, 463,000 new cases of breast cancer were diagnosed in 2012, and in the same period* ***131,000 deaths*** *were reported. Earlier and improved detection still requires a major effort.*

  The social, psychological and economic impacts on women, their families, friends and colleagues are incalculable, as are the healthcare and support costs are borne by society. Early and more sensitive diagnosis is crucial for this type of cancer.

- **Prostate Cancer** *is the most frequently diagnosed cancer among men accounting for nearly a quarter of all new male cancer diagnoses. More than 90% of all prostate cancers are diagnosed when the disease is limited to the prostate and surrounding organs. In Europe (2012) 416,000 new cases of prostate cancer were diagnosed in Europe with* ***92,000 registered deaths***.

The high rate of false negative results observed in first time prostate biopsies (15-30%), together with the high rate of false positives in PSA test (75%) significantly increases the number of unnecessary biopsies made worldwide.

The societal, psychological and economic impacts on the patients, their families, friends and colleagues are incalculable, as are the healthcare and support costs borne by society.

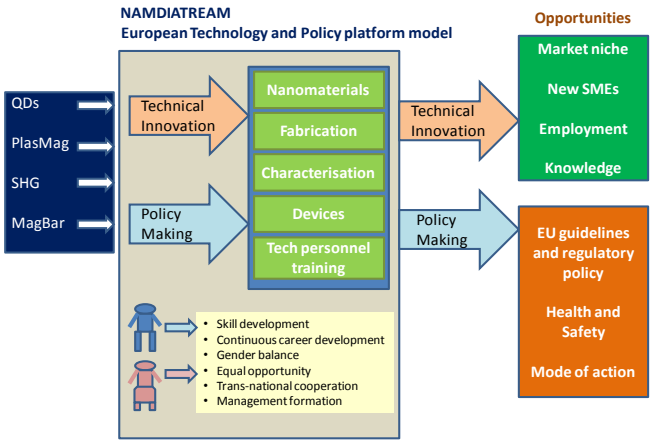
Therefore, ***NAMDIATREAM objectives were*** *focussed on* ***introducing and delivering super-sensitive diagnostic assays and devices to allow for the early diagnosis and monitoring of lung, breast and prostate cancers.***

The pressing needs for these have been *highlighted and extensively reported in the Joint European Commission/ETP Nanomedicine Expert Report 2009 on Roadmaps in Nanomedicine Towards 2020 (2013).*

Project Impact in relation to the objectives of the topic:

NAMDIATREAM project has delivered a number of innovative solutions based on nanotechnology for cancer detection and diagnostic imaging, see Scheme IMP2.1)

NAMDIATREAM OVERALL IMPACT STRATEGY



SCHEME IMP2.1. NAMDIATREAM overall impact strategy centred on the development of nanotools based on the four technological innovations, which aim at early diagnosis of three cancers type.

NAMDIATREAM consortium has fully benefited from the involvement of the world-recognised MNCs large industries, SMEs and research centres across different disciplines spanning from material science, physics, magnetism, chemistry, biochemistry, molecular biology, and medicine.

Measurable outcomes of this strategic alliance can be summarised below:

1) **SMEs and MNCs** have taken the evolving technology to a new level of readiness and maturity,

2) In total, a new employment stream has generated a total of 126 jobs (gender division: 33% female, 67% male) and of these, 108 jobs have been directly funded by NAMDIATREAM project,

3) NAMDIATREAM dissemination and exploitation activity exceeded the original expectation levels, which translated into a high value for money (over 130 peer-review publications, 290 conferences and seminars attended, more than 15 organised events (e.g. seminars, workshops, conferences and public events and 7 industry booths and fair trade presentations),

4) The engagement for the industrial partners was so active that 15 new job opportunities have been created.

This has additionally increased the competitiveness of the European partners involved in the IVD development.

European Approach: Implementation of the European Commission's Action Plan for Nanotechnology towards Horizon 2020

NAMDIATREAM through the extensive stakeholders engagement, as reported in the dissemination booklet published online, has promoted and contributed to the responsible introduction and safe adoption of nanotechnology in cancer diagnostics, prognostics and monitoring. Such engagement has been measured by the consortium partner's involvement into European Technology Platforms, Clusters, Technical Workgroups and Expert Committees towards the scientific and technical formulation of guidelines, roadmaps, white papers, perspectives and lead opinions.

Thus, NAMDIATREAM provided the basis of excellent science, industrial innovation, commercial focus to marketable products in the areas of diagnostic instrumentations, clinical devices, biomarkers development and point of care testing. This has been carried out in a purely technical and unbiased environment where the outputs of the project are to be trusted at all levels of society, giving reassurance if there are no disease implications identified, whilst providing an opportunity for responsible measures to protect citizens, should a particular hazard from engineered NP emerge.

European Approach: Sustainable and responsible development of Nanotechnology (securing the knowledge-based economy)

Public trust and acceptance of nanotechnology have been crucial for the project's long-term development and will allow the EU and its citizens to profit from its potential benefits. **This trust can be fostered by scientists that are independent and responsible researchers. We consider that outputs from the**

NAMDIATREAM project, which focuses on excellence in science and technical execution, combined with an educated approach to the development of new *in vitro* point of care and diagnostic devices, **will be a useful resource for regulatory agencies, and will serve the public need for responsible development of nanotechnology. In total, 9 organised and chaired workshops, 7 training schools, 2 conferences and 2 Pan-european nanomedicine events have been delivered within the 4 years of the NAMDIATREAM project.**

European dimension requirement and other national and international research activities

Technological breakthrough in the diagnostic sector needs to integrate different disciplines and requires a broad multidisciplinary scientific and technological approach. It was therefore difficult for one centre to possess expertise in all necessary disciplines; there is a general consensus that an integrated approach between many disciplines is indeed the key to success of both scientific and technological issues.

The integration of highly sensitive biomolecular techniques with **cutting-edge detection systems** has prompted a new paradigm in medical diagnostic and imaging. In particular, the project addressed key issues affecting the competitive position and growth of the European biomedical industry.

The NAMDIATREAM project, through an intensive network activity, has contributed to I) maturation **of the European IVD diagnostic sector** with associated competitive and sustainable growth, II) employment and III) cross-field training opportunities.

NAMDIATREAM Contribution is particularly remarkable for:

1. Development and (pre-clinically) validation of new nanotechnology-based solutions for molecular diagnostics and imaging, addressing the efficient disease management of tomorrow,
2. Setting technology platforms to increased knowledge of the effects between biological and non-biological entities, for better developed diagnostic and disease monitoring tools,
3. Providing the basis for better and more reliable diagnosis and identification of correct treatment regimens,
4. Broadening the applicability of the novel nanotechnology based toolkits and assays to both research and diagnostic environments,
5. Enabling scientists to obtain the relevant data with superior performing reagents, qualitatively new sensitivity at prognosis and monitoring level,
6. Offering the reduction of laboratory burden and results waiting time compared to the currently available methodologies,
7. Lowering costs and faster assays performance,
8. Offering perspectives for reducing long-term hospitalization incidence,
9. Advanced monitoring of treatment success and timely switching to other treatments, if required. By introducing NAMDIATREAM as an early and better monitoring solution, patients will have a better choice and access to **early stage treatment** with lower doses, **reduced side effects** and a wider choice of **treatment options.**

Finally, the success of NAMDIATREAM consortium is further witnessed by the fact that **several of its partners are already actively participating in a number of major national and international research programmes and networks**. This has allowed the consortium to ensure that the results of the project have been passed on and disseminated in the most efficient manner, as documented in the dissemination report and in Section 4.

Main dissemination and public awareness

The public awareness of the project achievements and dissemination activities has been raised to a qualitatively new level. More than 450 public events in the form of meetings, publications, media events,

trade shows and widely available web resources have been accumulated over the past four years of project operation.

Educationally the project has generated the first cohort of highly qualified experts in nanotechnology applications focused on diagnostics and monitoring of cancer. In total, four PhD and ten MSc degrees have been awarded to the students involved in the project.

NAMDIATREAM Dissemination booklet containing all NAMDIATREAM dissemination activity over the 4 years has been finalised and released in the public domain. The booklet aims to provide an overview of the multidisciplinary activities initiated by the Nanomedicine Research Group of Trinity College Dublin (Ireland) and its collaborating partners through the participation in the NAMDIATREAM (FP7 NMP LSP projects funded over the period 2009–2014), and also as part of its broader pan-European engagement.

Since the international and multidisciplinary dimensions of NAMDIATREAM went beyond the scope of the originally envisaged consortium activities, it also gathered other related projects and expertise by arranging outreach events in the fields of nanotechnology-enabled molecular imaging, therapeutics, and market strategies for nanomedicine. NAMDIATREAM dissemination booklet is available as open access document and is indexed as an ISBN publication. The Booklet is attached to this report in the appendix section.

Exploitation results:

Effective IPR management has led to the licensing of one of NAMDIATREAM generated IP to TIBIO Sagl, Comano (Switzerland) which in the third quarter of 2014 started the commercialisation of three NAMDIATREAM nano-products. These probes based on non-centrosymmetric harmonic nanoparticles have been developed during the course of project for the Biomedical and Optical Spectroscopy market.

Strategic exploitation plan has been also developed across the 4 vertical and 1 horizontal TPs in order to explore any possible transfer of know-how developed to the market, and it extends beyond the lifespan of NAMDIATREAM.

CONCLUSIONS

The technologies developed by NAMDIATREAM have been integrated into demonstration diagnostic systems operating on the principles of optical, plasmonic, non-linear optical and magnetic detection of cancer cells and molecular markers. The performance of the new systems proved to be comparable or superior to the quality obtained with gold standard protocols of diagnostic practice.

The project has provided a significant additional value for money for the stakeholders. Several technological achievements have been either carried out into a wider exploitation context or transferred into commercial settings.

Fundamental scientific achievements and breakthroughs served as the basis for new and follow-up projects funded by:

- ***i) National and international strategic research and commercial grants,***
- ***ii) Horizon 2020 KETs, LEIT, NMP, Health, and SME instrument funding opportunities,***
- ***iii) Infrastructural and core facility supporting funding instruments,***
- ***iv) European and regional development funding opportunities for SMEs.***

Public awareness of the project achievements and dissemination activities has been maintained through more than 450 events in the form of meetings, publications, media coverage, trade shows and web resources throughout the project lifespan. The international and multidisciplinary dimensions of NAMDIATREAM went beyond the scope of the originally envisaged consortium activities and it also gathered other related projects and expertise by arranging outreach events in the fields of nanotechnology-enabled molecular imaging, therapeutics and market strategies for nanomedicine.

Educationally, the project has generated the first cohort of highly qualified experts in nanotechnology applications focused on diagnostics and monitoring of cancer.

Overall, the NAMDIATREAM consortium has significantly contributed to the scientific progress and technological advances in biomedical sciences along with the breakthrough clinical applications achievements in the areas of Cancer, Diagnostics, Healthcare, Nanotechnology, Nanomedicine, Smart systems for Healthcare and other relevant fields, for the ultimate benefit of the European stakeholders.

Public website address

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