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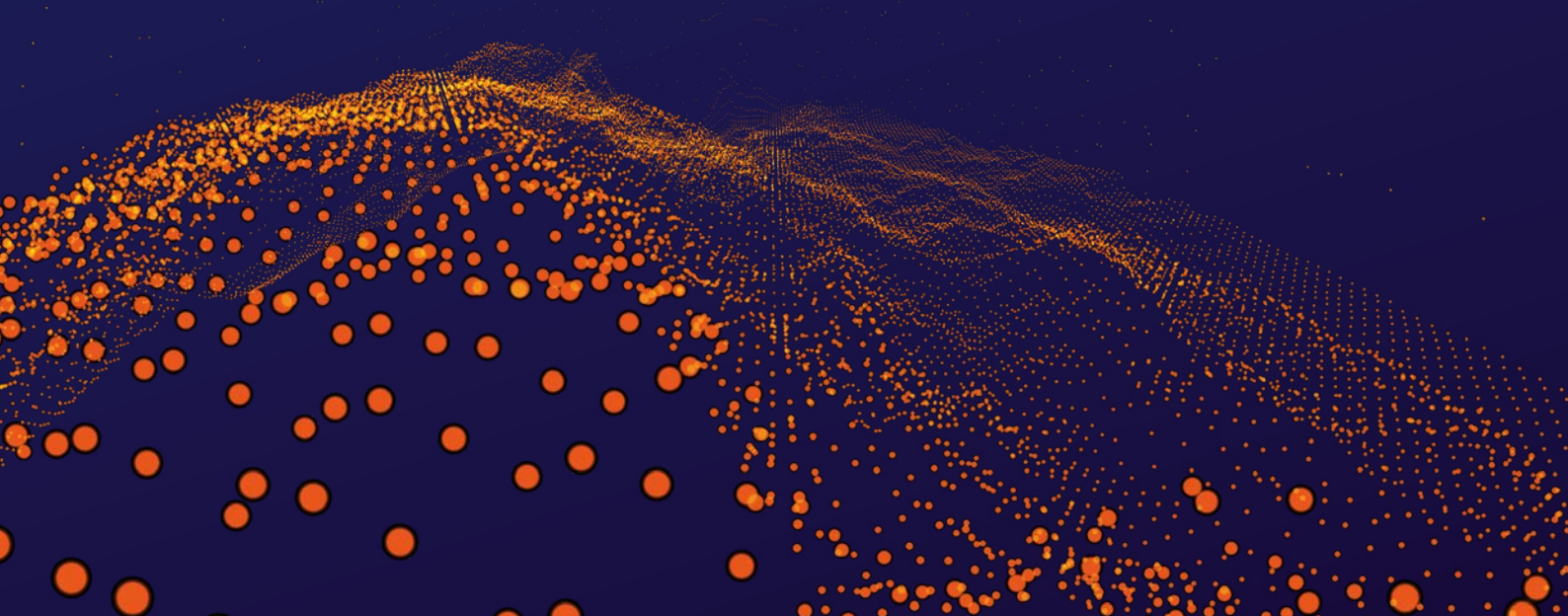
NPOT® technology for drug target identification

White paper



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The importance of identifying targets

Insufficient validation of drug targets at an early stage has been linked to costly clinical failures and low drug approval rates[1,2].Therefor target identification is a crucial step in drug development. Indeed, knowing the target enables the elucidation of the drug mode of action, the identification of triggered pathways, eventual side-effects and further characterization of detailed drug-target interactions. The sooner the drug target is identified the more efficient drug development will be. It is crucial for adequate drug development and defines its most relevant applications.

"Early target identification accelerates drug development"

This white paper discusses the current classic methods of drug-target identification and how label-free **Nematic Protein Organisation Technique (NPOT®)** can secure, accelerate and improve the process of drug development.

Currently available methods for drug identification

Compared to target-based screening, phenotypic screening constitutes an advantage for the discovery of first-in-class therapeutics[3]. However, in this context, the identification of the direct targets constitutes a major drawback in elucidating the underlying mechanism of action. Conventionally, the

identification of drug targets has been performed using standard genetic and biological experimental techniques. As an example, hypotheses regarding the mechanism of action of a compound can be generated by gene expression profiling in the presence or absence of compound treatment[4]. In the last years, pull-down assays have also been widely used to identify potential binding partners by either activity-based or affinity-based proteome profiling[5]. This chemo-proteomic approach requires however changes of the chemical structure of interest, by adding an affinity tag or probe for immobilization to the support. Such modifications might modify the drug-target interactions and might lead to inadequate identification. To overcome such difficulties, other approaches have been developed. They include machine learning methods, where several biological databases can be used in combination with different algorithms to predict potential interactions[6–8]. Usually, using machine learning methods for predicting protein drug targets follow three steps: first, the selection of the datasets and features; second, selection of the learning algorithms, and finally, the assessment of the prediction performance of the models[9]. Although machine learning appears revolutionary, machine learning methods based on in silico studies and available databases, show limited accuracy and specificity. Current methods, both biological and computational, still have many limitations[9]. Better solutions, from both biological and computational sides, must be developed.

"Label-free NPOT® technology can secure, accelerate and improve the process of drug development"

For this reason, NPOT®, known as Nematic Protein Organisation Technique, has been developed. This interactomic method presents the main advantage of identifying targets under physiological and/or pathological conditions, together with their native interactome.

NPOT®: a new method for target identification

Concept

NPOT® is a label-free proprietary technology that enables the direct isolation and identification of specific macromolecular scaffolds implemented in healthy or pathological conditions directly from human tissue or cell lines. It is based on Kirkwood-Buff molecular crowding[10,11] and aggregation theory[12,13].

How it is performed?

Concretely, a molecule of interest (small molecule, peptide or antibodies) is mixed with the protein content from a cell line, body fluid or tissue which have been prepared without any detergent to maintain initial protein and molecular interactions. The resulted mixture is then placed in a liquid pH gradient miming the one found in our cells, i.e., both acid and basic. It covers pH from lysosomes to mitochondria. In such gradient proteins interacting with the test compound will precipitate out as they reach their zwitterion point. The latter will allow the formation of subsequent heteroassemblies, which are then solubilized and analyzed through nano liquid chromatography-mass spectrometry (LC-MS/MS). The peptides are then identified and quantified by tandem mass spectrometry. The whole NPOT® process is described in Figure 1.

"Label-free NPOT® technology enables the target identification without any labeling and in physiological conditions"

Importantly, this procedure is conventionally performed in parallel with a negative control compound, that could be an inactive enantiomer or a compound with a close chemical structure to the test compound with less or no pharmacological activity. This negative control enables the identification of selective and specific proteins within the interactome.

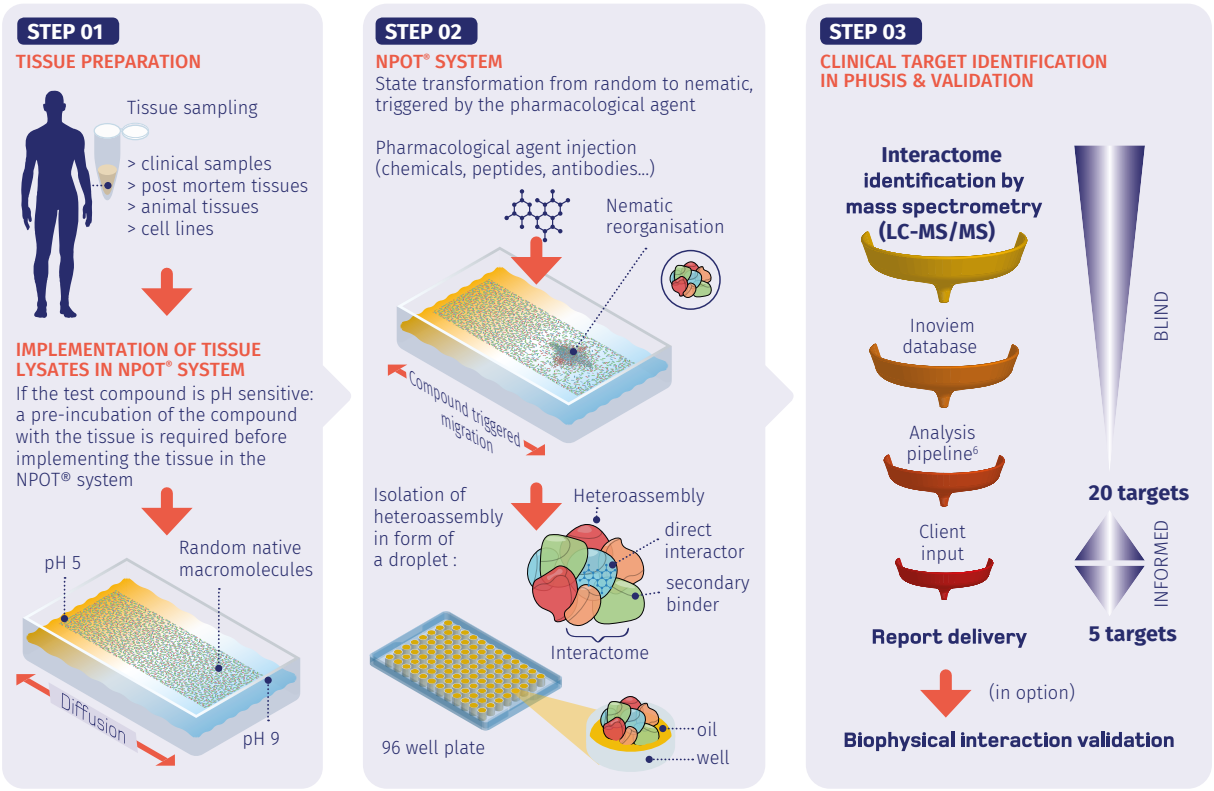

Figure 1. Description of the NPOT® process from tissue/cell preparation to target identification.

NPOT® heteroassemblies visualization

As shown in Figure 2 and Figure 3, the shape of the resulting heteroassemblies might differ according to the type of tissues and molecules used to perform the NPOT®. Importantly, changes are always observed in the protein content of each NPOT® heteroassembly depending on the molecule used in the same tissue. The specifically identified proteins within each interactome are thoroughly analyzed.

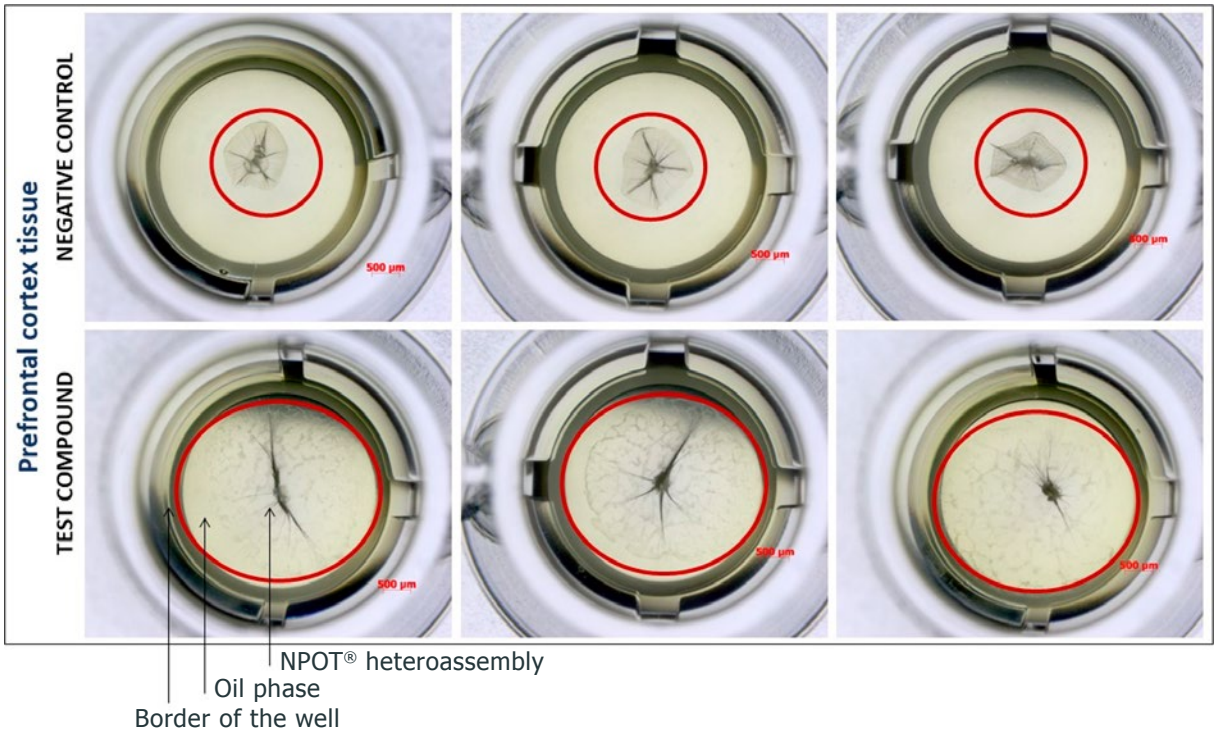

Figure 2. Pictures of the heteroassemblies obtained by NPOT® on human prefrontal cortex tissue, in presence of a negative control compound (upper panel) and a test control compound (lower panel). Please note that for the same tissue, slight to moderate changes in the shape of the heteroassemblies are observed according to the molecule used.

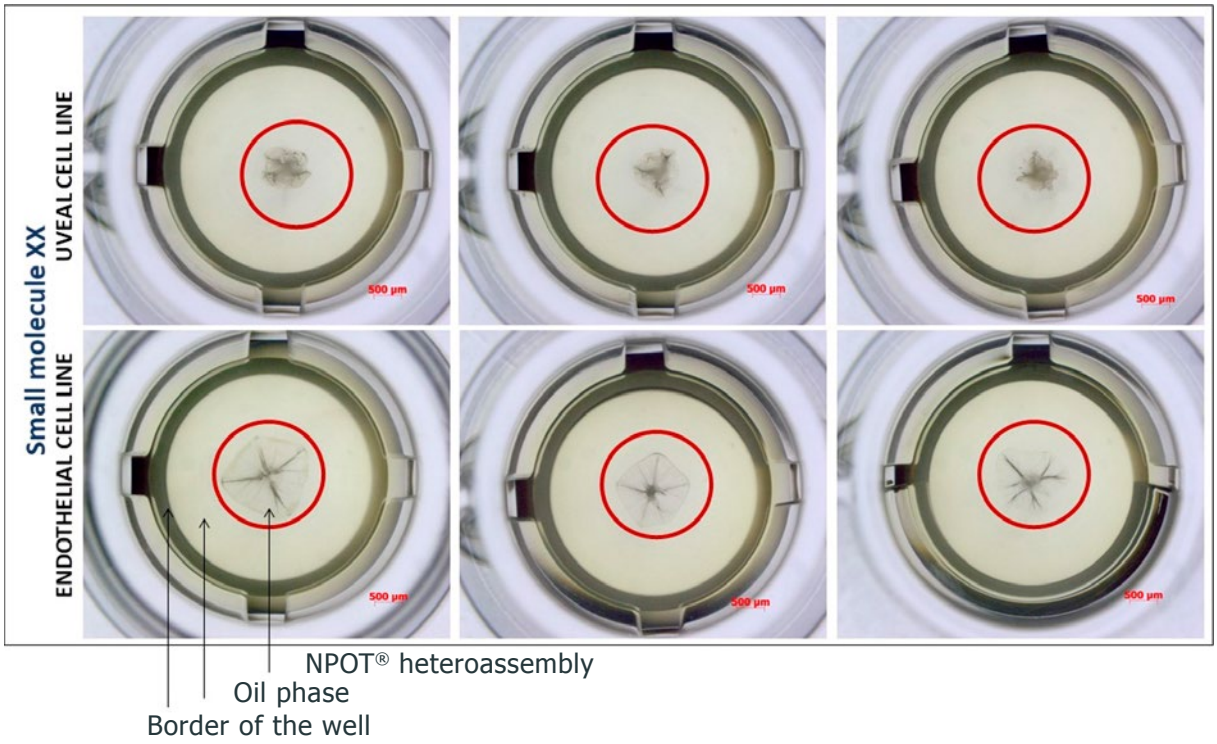

Figure 3. Pictures of the heteroassemblies obtained by NPOT® on two different cell lines with a small molecule (XX). NPOT® performed on a uveal cell line and on an endothelial cell line are shown respectively in the upper panel and lower panel.

What does NPOT® deliver?

It delivers from any patient cohort or any pharmacological model the following three **enabling information**:

1. Primary target candidates
2. Functional signaling pathway
3. Compound mode of action

Analysis process

The analysis process is composed of 3 steps:

1. Discrimination between specific proteins and nonspecific proteins
2. Discrimination between targets, indirect effectors and frequent hits
3. Analysis of the enriched pathways

→ Specific protein interactome identification

Identification of specific interactome

The protein content of each heteroassembly is specific to each tissue/molecule, and is composed of direct targets, including ON- and OFF-targets, indirect effectors, but also nonspecific proteins. The analysis is crucial to enable the distinction of each entity. Nonspecific proteins, i.e. proteins that coprecipitate due to their abundancy, or proteins that are frequently identified over different drug-mediated NPOT® interactomes (considered as frequent hits), are identified by two different manners: (i) by comparing the dataset of the NPOT® performed with the negative compound; (ii) by comparing their frequency of appearance within a large set of NPOT® data. To make such calculation possible, Inoviem Scientific developed its own application, called InOPERA®, that calculates the occurrence of one given protein in the entire database, or specific datasets matching defined criteria of species, tissues or cell lines. InOPERA® distinguishes nonspecific proteins (proteins which displayed a high occurrence within the InOPERA® database), from specific proteins, which include the target. As an example of nonspecific protein, actin or tubulin, known to be abundant in cells, have a high percentage. Targets and indirect effectors would display lower InOPERA® frequencies.

Enriched pathways analysis: "finding the way to targets"

To identify the enriched pathways within each interactome, these specific and low InOPERA® frequency protein lists are then analyzed using different bioinformatics resources, highlighting the enrichment of biological processes and pathways in a given protein list based on infor¬mation in Gene Ontology[14] (GO) and pathways databases (KEGG[15], Reactome[16]...). The specifically identified proteins should form or be part of a relevant pathway.

Table 1. The set of diverse knowledgebases that provides a current biological understanding of the NPOT® interactome; these data sources are integrated and applied to support the analysis and the visualization of NPOT®.

Application programming interface	Associated databases used for NPOT® analysis
InOPERA®	Proprietary NPOT® information
Resources for enrichment pathway analysis	GO (Gene Ontology: Biological Process) KEGG REACTOME Protein Atlas UniProt DrugBank BioGRID Biocarta BioCyc
Resources for protein interaction analysis	BIND DIP GRID HPRD IntAct MINT PID

The successful target identification is exemplified in several studies[17–20] and covers several fields of applications (Figure 4).

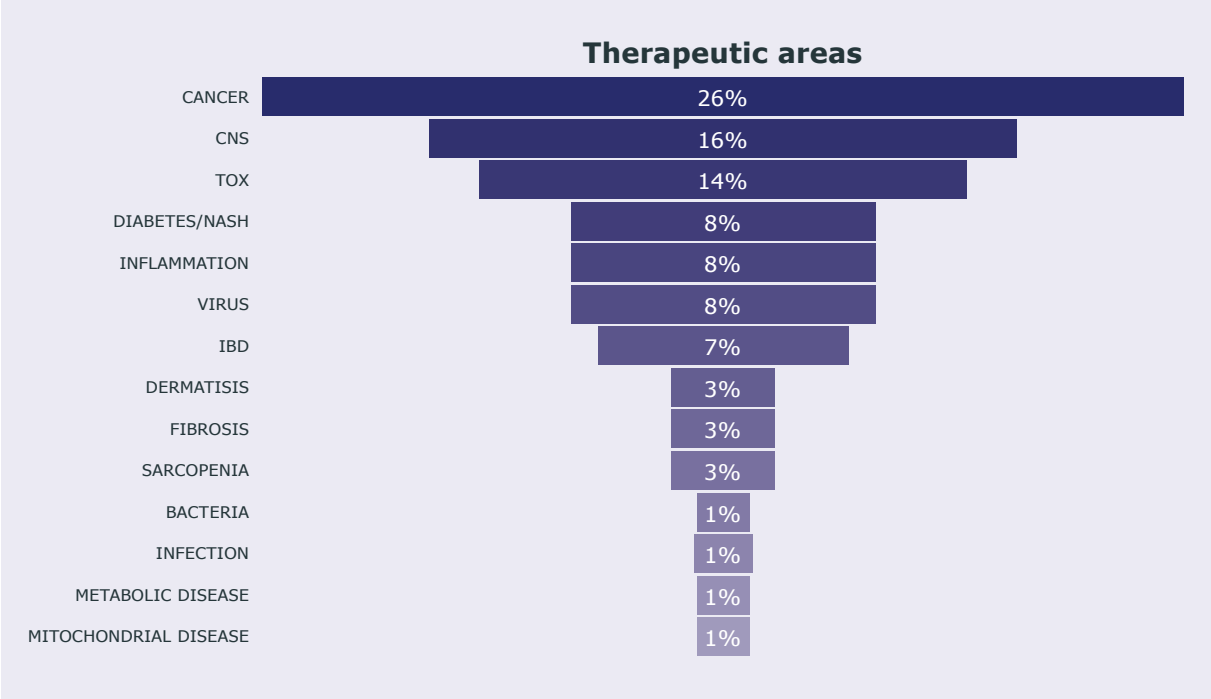

Figure 4. Chart representing the different fields in which NPOT® technology was applied.

Target Validation

While target identification is challenging and require innovative technologies, target validation can be performed using standard biological approaches, including genetic (knockdown), molecular biology with various competition assays or pharmacology. However, these approaches are sometimes tedious and direct target-drug assays can be performed to quickly determine the possible interaction between two entities. Indeed, thermal stability assay (TSA) and in its cellular variant CETSA (Cellular Thermal Shift Assay), can be used to address the question of direct interaction. In this test, the thermal stability shift is used to validate the ligand engagement[21]. However, these methods rely on the quality and reliability of antibodies, and are not optimal for membrane proteins[22].

To overcome these limitations, surface plasmon resonance (SPR) can be performed to quickly assess the possible interaction between a drug and its potential target[23]. SPR is an optical technique for detecting the interaction of two different molecules in which one is mobile and one is fixed on a chip composed of dextran and a thin gold film[24]. This method can be applied on both membrane and cytosolic proteins and assesses the binding specificity, active concentration measurements, and the determination of kinetic and thermodynamic parameters[23]. Further technologies, such as nanoDSF (nano Differential Scanning Fluorimetry) or Microscale Thermophoresis (MST) can also be used to identify the specificity of an interaction and interaction dynamics or screen different ligands[25].

"Biophysical approaches facilitate the direct assessment drug-target interactions"

Summary

NPOT® technology is **a novel technology that enables the identification of drug targets and the underlying mechanism of any compound**. Compared to other label-free methods, NPOT® is performed in **physiological environments, in any biological samples or directly on human tissue both healthy and diseased**.

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