

FKZ: 16GW0365

HIVacToGC Project: Final Report Part I

Project Overview

Title: HIV Vaccine Targeting via DNA Origami Nanoparticles to Lymph Nodes to Promote Germinal Center Formation (HIVacToGC)

Duration: 36 months

Objectives

The HIVacToGC project was conceived to advance HIV vaccine development by using DNA nanotechnology and high-resolution, whole-organism imaging techniques. The specific objectives were to design and synthesize DNA origami nanoparticles (DONPs) capable of efficiently delivering HIV immunogens to target lymphoid tissues; develop advanced imaging and analysis pipelines for the comprehensive assessment of nanocarrier biodistribution at single-cell resolution across entire mouse bodies; evaluate the efficacy of DONP-based vaccine candidates in promoting germinal center formation within lymph nodes; investigate potential off-target effects and safety profiles of the developed nanocarriers using high-sensitivity whole-body analysis techniques; and establish a generalizable platform for the rapid assessment and optimization of nanocarrier-based therapeutics, with potential applications beyond HIV vaccine development.

Project Adaptation

Due to technical delays in producing the originally planned DNA origami nanoparticles within the consortium, we strategically broadened our approach to assess various nanocarrier systems. This adaptation allowed us to develop and validate our advanced imaging pipelines while exploring the potential of different nanocarrier types. We systematically evaluated silica nanoparticles (SiNPs), lipid nanoparticles (LNPs), and other nanocarrier formulations, each offering unique insights into delivery mechanisms and biodistribution patterns. This comprehensive assessment of multiple nanocarrier systems proved invaluable, enabling us to establish robust, versatile imaging and analysis techniques applicable to various nanocarrier types. The expanded scope of our research addressed the immediate challenges posed by the technical delays and significantly enhanced our findings' broader applicability. As our imaging pipelines matured and demonstrated their effectiveness across various nanocarrier systems, we gradually refocused our efforts on the original HIV vaccine development goals. The insights from studying diverse nanocarriers, particularly the highly efficient LNPs, informed our approach to optimizing DNA origami nanostructures for HIV immunogen delivery. This adaptive strategy allowed us to make substantial progress on our core objectives while simultaneously developing tools and knowledge with far-reaching implications for the field of nanomedicine.

Key Results

1. **Advanced Imaging and Analysis Pipelines:** We successfully developed two complementary, cutting-edge pipelines: DELiVR (Deep Learning and Virtual Reality Mesoscale Annotation Pipeline) and SCP-Nano (Single Cell Precision Nanocarrier Identification Pipeline). These pipelines integrate virtual reality annotation, deep learning algorithms, optimized tissue clearing techniques, and light-sheet microscopy to enable

rapid and accurate detection of cellular targets and nanocarrier distribution at single-cell resolution throughout entire mouse bodies.

2. **Enhanced Detection Sensitivity:** The SCP-Nano pipeline demonstrated unprecedented sensitivity in detecting nanocarriers, visualizing and quantifying lipid nanoparticles (LNPs) at doses as low as 0.0005 mg/kg, a 100-1000-fold improvement over conventional imaging methods, allowing for a more accurate assessment of nanocarrier distribution at clinically relevant doses.
3. **Comprehensive Nanocarrier Distribution Analysis:** We successfully visualized and quantified the distribution of various nanocarriers, including LNPs, DNA origami structures, and adeno-associated viruses (AAVs), revealing distinct distribution patterns based on administration routes. The high-resolution, whole-body analysis enabled the detection of sparse, off-target accumulation, highlighting the importance of sensitive whole-body analysis in nanocarrier development.
4. **Proteomic Analysis of Off-Target Effects:** In regions of off-target LNP accumulation, particularly in heart tissue, proteomic analyses revealed alterations in the expression of inflammatory and vascular proteins, aligning with certain clinical observations and underscoring the critical role of comprehensive, high-sensitivity analyses in elucidating potential side effects of nanocarrier-based therapeutics.
5. **Versatility and Broad Applicability:** The developed imaging pipelines demonstrated remarkable versatility and were successfully applied to detect various cellular markers and analyze multiple nanocarrier types. This broad applicability suggests that our platform could be adapted to various therapeutic applications beyond the initial scope of HIV vaccine development.
6. **Insights into Germinal Center Formation:** While the primary focus shifted towards developing advanced imaging techniques, our findings provide a robust foundation for future studies on germinal center formation, offering unprecedented opportunities to optimize vaccine design for enhanced immune responses.

Conclusion

The HIVacToGC project has made substantial contributions to the field of nanocarrier-based therapeutics. The development of DELiVR and SCP-Nano pipelines represents a significant advancement in our ability to analyze nanocarrier distribution and effects at single-cell resolution throughout organisms. These tools promise to accelerate the development of precise and safe nanocarrier-based therapeutics by providing comprehensive insights into biodistribution and potential off-target effects at unprecedented resolution and sensitivity. While the project's scope expanded beyond its initial focus on HIV vaccines, the resulting technologies and insights have broad implications for nanomedicine and drug delivery fields.

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HIVacToGC Project: Final Report Part II

1. Introduction and Project Overview

The HIVacToGC project (HIV Vaccine Targeting via DNA Origami Nanoparticles to Lymph Nodes to Promote Germinal Center Formation) was conceived to advance HIV vaccine development through the innovative application of DNA nanotechnology and high-resolution, whole-organism imaging techniques. Over 36 months, our consortium aimed to design and synthesize DNA origami nanoparticles (DONPs) capable of efficiently delivering HIV immunogens to target lymphoid tissues, develop advanced imaging and analysis pipelines for comprehensive assessment of nanocarrier biodistribution at single-cell resolution across entire mouse bodies, evaluate the efficacy of DONP-based vaccine candidates in promoting germinal center formation within lymph nodes, investigate potential off-target effects and safety profiles of the developed nanocarriers using high-sensitivity whole-body analysis techniques, and establish a generalizable platform for the rapid assessment and optimization of nanocarrier-based therapeutics.

2. Project Adaptation and Expanded Scope

Due to technical delays in producing the originally planned DNA origami nanoparticles within the consortium, we strategically broadened our approach to assess various nanocarrier systems. This adaptation allowed us to develop and validate our advanced imaging pipelines while exploring the potential of different nanocarrier types. We systematically evaluated silica nanoparticles (SiNPs), lipid nanoparticles (LNPs), and other nanocarrier formulations, each offering unique insights into delivery mechanisms and biodistribution patterns. This comprehensive assessment of multiple nanocarrier systems proved invaluable, enabling us to establish robust, versatile imaging and analysis techniques applicable to various nanocarrier types. The expanded scope of our research addressed the immediate challenges posed by the technical delays and significantly enhanced our findings' broader applicability. As our imaging pipelines matured and demonstrated their effectiveness across various nanocarrier systems, we gradually refocused our efforts on the original HIV vaccine development goals.

3. Key Results and Achievements

3.1 Development and Optimization of DISCO clearing and imaging and Analysis Pipelines

We successfully optimized our clearing/ imaging technologies for nanoparticle evaluation in mouse whole bodies and developed AI-based complementary, cutting-edge pipelines:

1. DELiVR (Deep Learning and Virtual Reality Mesoscale Annotation Pipeline)
2. SCP-Nano (Single Cell Precision Nanocarrier Identification Pipeline)

These pipelines integrate virtual reality annotation, deep learning algorithms, optimized tissue clearing techniques, and light-sheet microscopy to enable rapid and accurate detection of cellular targets and nanocarrier distribution at single-cell resolution throughout entire mouse bodies. These systems demonstrated unprecedented sensitivity in nanocarrier detection (0.0005 mg/kg), representing a 100-1000x improvement over traditional methods.

3.3 Comprehensive Nanocarrier Distribution Analysis

We successfully visualized and quantified the distribution of various nanocarriers, including LNPs, DNA origami structures, and adeno-associated viruses (AAVs), revealing distinct distribution patterns based on administration routes. The high-resolution, whole-body analysis enabled the detection of sparse, off-target accumulation, highlighting the importance of sensitive whole-body analysis in nanocarrier development.

3.4 Proteomic Analysis of Off-Target Effects

In regions of off-target LNP accumulation, particularly in heart tissue, proteomic analyses revealed alterations in the expression of inflammatory and vascular proteins, aligning with certain clinical observations and underscoring the critical role of comprehensive, high-sensitivity analyses in elucidating potential side effects of nanocarrier-based therapeutics.

3.5 Versatility and Broad Applicability

The developed imaging pipelines demonstrated remarkable versatility and were successfully applied to detect various cellular markers and analyze multiple nanocarrier types. This broad applicability suggests that our platform could be adapted to various therapeutic applications beyond the initial scope of HIV vaccine development.

3.6 Insights into Germinal Center Formation

While the primary focus shifted towards developing advanced imaging techniques, our findings provide a robust foundation for future studies on germinal center formation, offering unprecedented opportunities to optimize vaccine design for enhanced immune responses.

4. Detailed Methodology and Results

4.1 Development and Optimization of DISCO Clearing and Imaging Pipeline

The technical development focused first on optimizing fluorophore combinations for multi-channel imaging. We conducted extensive testing with various Alexa fluorophores (488, 567, 647, and 750) to determine optimal spectral combinations that minimize crosstalk while maximizing signal detection. This process involved careful calibration of detection parameters and assessment of signal stability throughout the tissue-clearing process. A critical component of our pipeline development involved integrating the vDISCO clearing protocol with methods for preserving fluorescent protein signals. We refined our approach to maintain signal integrity while achieving the transparency needed for whole-body imaging. The protocol incorporated signal amplification using GFP nano boosters, which proved essential for achieving the sensitivity required for tracking nanoparticle distribution at the cellular level. We established rigorous quality control parameters throughout the imaging workflow, including optimizing signal-to-noise ratios across different tissue types, measuring tissue penetration depth, and benchmarking resolution at both cellular and subcellular scales. Light-sheet microscopy parameters were carefully tuned to enable whole-body imaging while maintaining the resolution necessary for single-cell analysis.

The resulting standardized protocols (**Fig. 1**) provide a robust foundation for future nanoparticle distribution studies, ensuring consistent, high-quality imaging across different tissue types and fluorescent markers. This technical groundwork puts us to efficiently execute the planned

nanoparticle distribution studies with maximum sensitivity and cellular resolution once the materials become available.

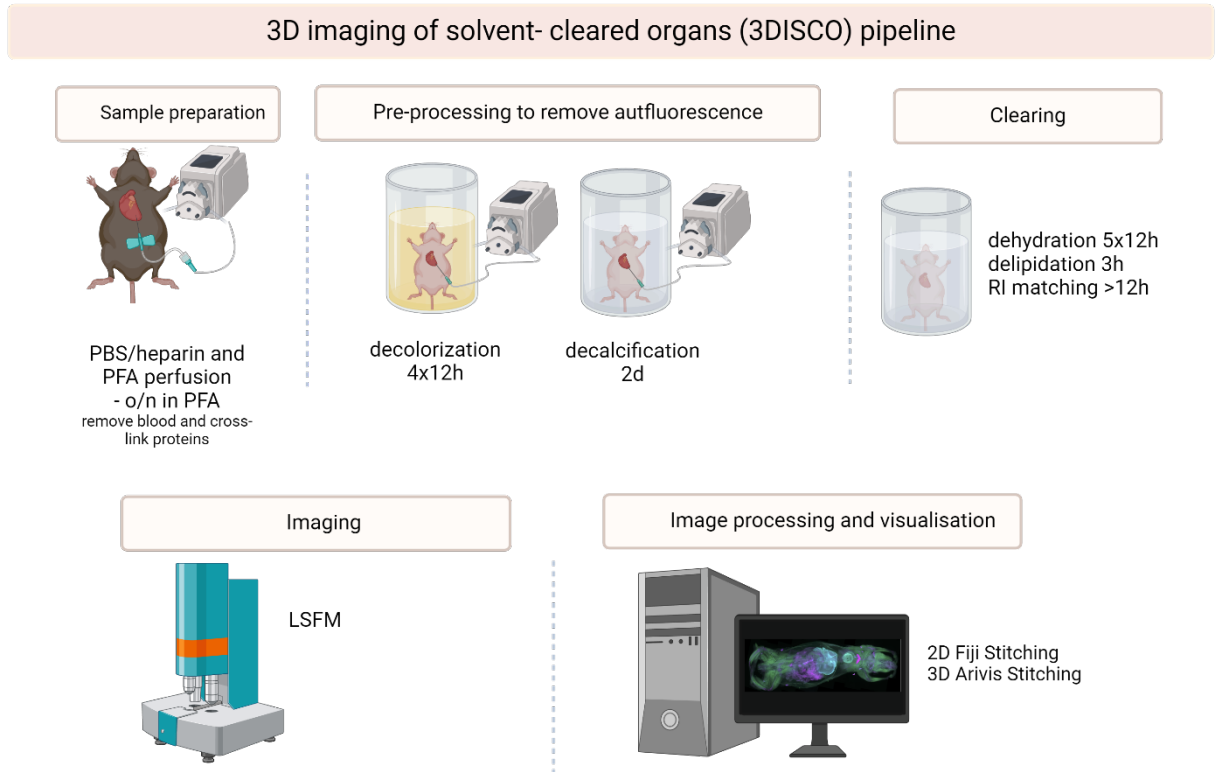


Fig. 1: DISCO Clearing and Imaging Pipeline: Mice are subjected to intracardiac perfusion to remove the blood and cross link the proteins to fix the tissues. The whole mouse bodies are decolorized and decalcified before optical clearing and imaging using light sheet microscopy. Collected images are processed using Fiji and Arivis software for 3D rendering.

Development and Optimization of Analysis Pipelines

4.1.1 DELiVR Pipeline

The DELiVR pipeline combines virtual reality (VR) annotation with deep learning algorithms to enable efficient and accurate analysis of large-scale, high-resolution imaging data. This pipeline addresses the challenges associated with manual annotation of complex 3D datasets by leveraging the intuitive interface of VR technology.

Key components of the DELiVR pipeline include:

1. VR-based annotation tool for efficient labeling of cellular structures and nanoparticles
2. Deep learning models trained on VR-annotated data for automated segmentation and classification
3. Integration with tissue clearing and light-sheet microscopy data acquisition workflows

The DELiVR pipeline significantly reduced the time required for data annotation while maintaining high accuracy, enabling the analysis of whole-body datasets that were previously intractable due to their size and complexity.

4.1.2 SCP-Nano Pipeline

The SCP-Nano pipeline was developed to achieve single-cell precision in nanocarrier identification and quantification across entire mouse bodies. This pipeline builds upon the tissue clearing and imaging techniques established in our lab, incorporating advanced deep-learning algorithms for nanoparticle detection and cellular context analysis.

Key features of the SCP-Nano pipeline include:

1. Optimized tissue-clearing protocols for whole-body imaging
2. High-resolution light-sheet microscopy acquisition
3. Deep learning-based nanoparticle detection and segmentation
4. Cellular context analysis for precise localization of nanoparticles within tissues
5. Quantitative analysis tools for assessing nanocarrier biodistribution and accumulation

The SCP-Nano pipeline enabled us to achieve unprecedented sensitivity in nanocarrier detection, visualizing particles at concentrations orders of magnitude lower than previously possible.

4.2 Nanocarrier Biodistribution Studies

4.2.1 Acute Silica Nanoparticles (SiNPs) biodistribution

In the absence of DNA origami nanoparticles, we initially focused on characterizing the biodistribution of silica nanoparticles (SiNPs) functionalized with the HIV vaccine candidate Env immunogen. Male BalB/C mice (6 weeks old) were subcutaneously injected with Env-SiNPs (8 μ g protein concentration) labeled with one or two fluorophores (Atto568 on SiNPs and Atto647 on Env peptide). The experimental design (**Fig. 2**), including animal administration and imaging protocols, was developed internally. Following subcutaneous injection, the nanoparticles were left to circulate in the mouse body 24 hours before the mice underwent perfusion and optical tissue

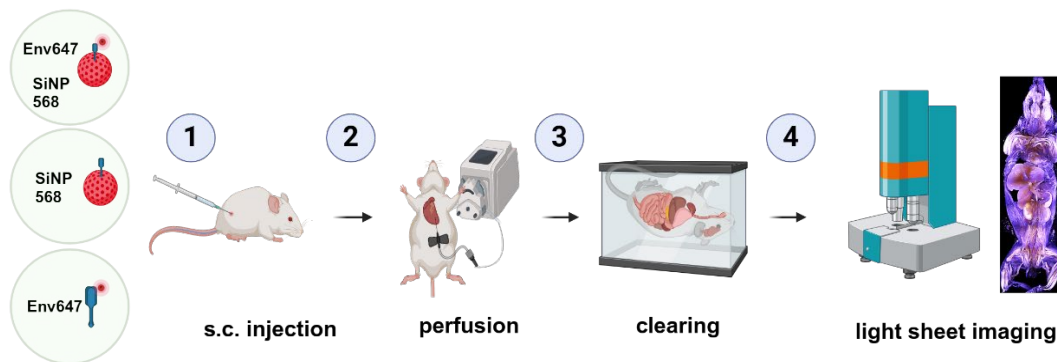


Fig. 2: Workflow for biodistribution analysis of nanoparticles (NPs): Fluorescent NPs with or without Env conjugation and the Env alone are (1) injected subcutaneously into mice and allowed to circulate in the body. (2) The animals are then perfused, and their entire body (3) is subjected to optical clearing, followed by (4) light sheet microscopy examination.

clearing. Whole-body imaging was performed using a custom-made light sheet microscope with a large chamber to accommodate sizable samples, such as whole mice, for imaging purposes.

We found that the SiNP signals were mainly visible at the injection site at low resolution, like the Env alone. Both fluorophores were co-localized, suggesting that at least part of the cross-linking between the SiNPs and the Env is stable in vivo (**Fig. 3**).

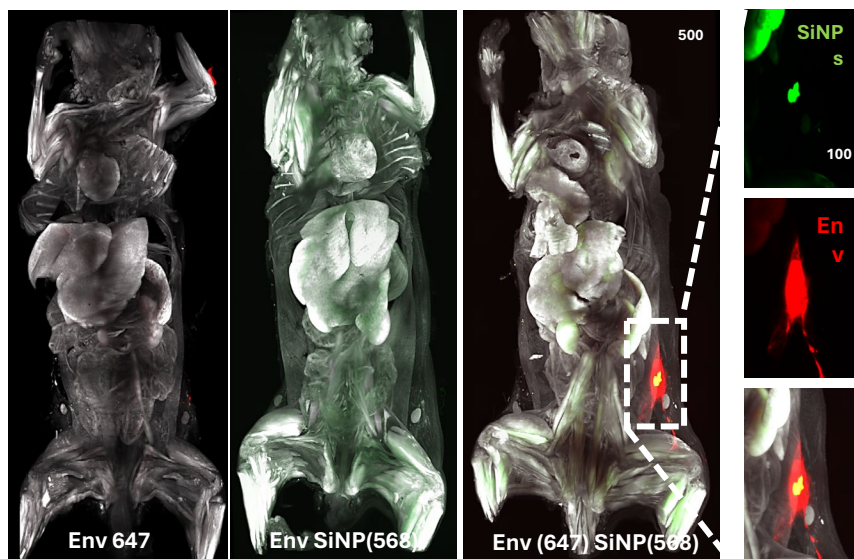


Fig. 2: Workflow for the acute biodistribution analysis of nanoparticles (NPs): fluorescent SiNPs with or without Env conjugation and the Env alone were injected subcutaneously into the mice and allowed to circulate for 24 hours. The SiNPs were coupled with the dye Atto568, while the Env peptide was labeled with the dye Atto 647. Imaging was performed using light sheet microscopy.

Key findings from the SiNP studies include:

1. Predominant localization of SiNPs at the injection site 24 hours post-administration
2. Co-localization of both fluorophores, suggesting stable cross-linking between SiNPs and Env in vivo
3. Weak signals were detected in the liver, kidneys, and lungs at high resolution, with non-colocalized Env and SiNP signals
4. Large aggregates of Env signal observed in various tissues
5. No obvious specific signals were detected in targeted lymph node tissues

4.2.2 Long-term Silica Nanoparticles (SiNPs) biodistribution

We conducted an extended biodistribution study to evaluate the temporal dynamics of nanoparticle distribution and Env-protein localization. This experiment included two groups: one receiving Env-SiNPs alone and another receiving Env-SiNPs with MPLA adjuvant. Circulation times were 4 hours, three days, seven days, three weeks, and ten weeks before

mice were perfused and tissues were cleared for imaging, mirroring our previous experiment (Fig. 3).

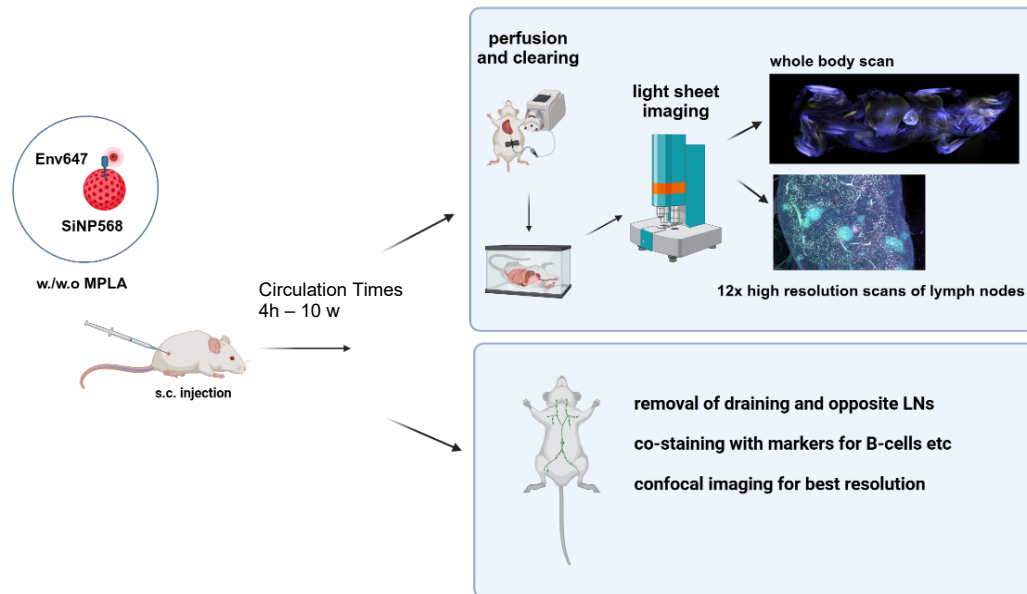


Fig. 3: Workflow for long term biodistribution analysis of nanoparticles (NPs): fluorescent SiNPs with double fluorescent tag (SiNPs were coupled with the dye Atto568, while the Env peptide was labeled with the dye Atto 647) with or without MPLA adjuvant were injected subcutaneously into the mice and allowed to circulate for different times ranging from 4h to 10 weeks. Mice were split in two groups. The first group was subjected to whole body imaging using light sheet microscopy. The second group was processed to extract the lymph nodes for confocal microscopy.

Our findings reveal distinct temporal dynamics in nanoparticle distribution and Env-protein localization. After one day, the initial presence of nanoparticles is observed in the lymph nodes, predominantly concentrated on the exterior. By day 3, a significant accumulation of nanoparticles is observed within the lymph nodes proximal to the injection site, coinciding with free Env-protein in the surrounding tissue. Notably, after three weeks, concentrated areas of Env-protein emerge within the lymph nodes adjacent to the injection site, suggestive of germinal center formation. Interestingly, this phenomenon is exclusively observed in mice receiving SiNP Env without MPLA adjuvant (Fig. 4). In addition to nanoparticle imaging, we also screened antibodies against the Env peptides for immunostaining of lymph nodes, enabling validation of Env-SiNPs distribution at cell and sub-cellular resolution. These antibodies were produced within the consortium.

Key observations from the long-term biodistribution studies:

1. Significant accumulation of nanoparticles within proximal lymph nodes by day 3, coinciding with free Env-protein in the surrounding tissue
2. Emergence of concentrated areas of Env-protein within lymph nodes adjacent to the injection site after three weeks, suggestive of germinal center formation (observed only in mice receiving SiNP Env without MPLA adjuvant)

These findings underscore the complex interplay between nanoparticle circulation time, adjuvant presence, and Env-protein localization within lymph nodes, providing valuable insights for optimizing nanoparticle-based vaccine delivery strategies. Earlier this year, We presented and discussed these findings at the Keystone Symposia on Delivery of Nucleic Acid Therapeutics.

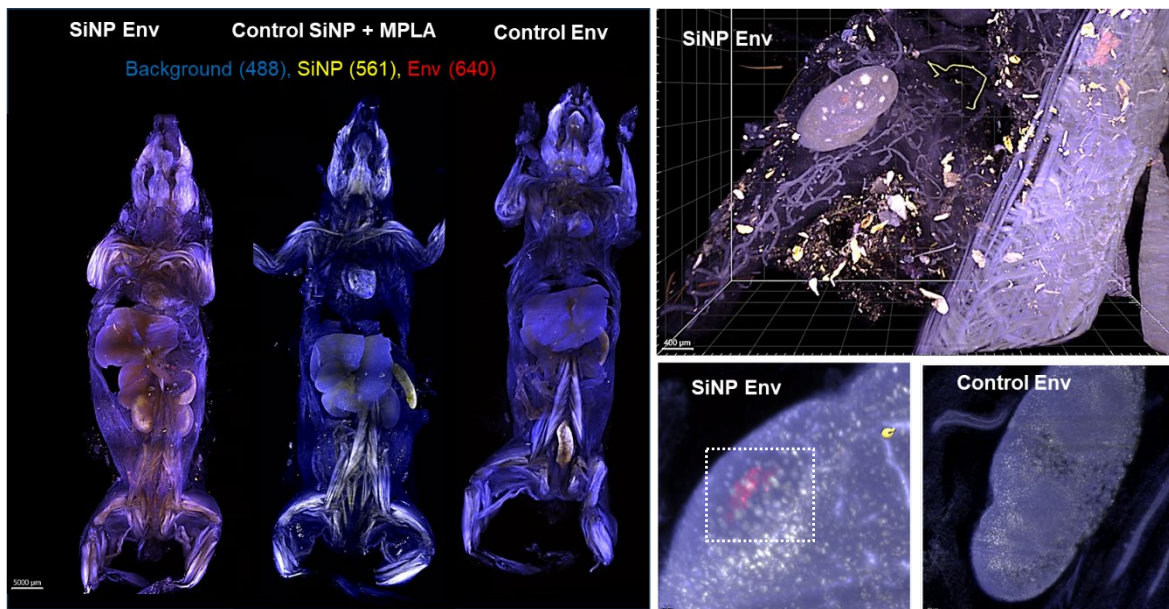


Fig. 4: Illustration of biodistribution analysis of SiNPs after 3 weeks circulation: Whole body imaging showing the SiNPs (SiNP Env) and two controls (SiNPs without the Env peptide injected with MPLA adjuvant and the Env peptide injected alone). A zoom out of the lymph node next to the injection site from a mouse injected with the SiNPs and the control Env alone imaged at higher resolution (12X) using light sheet microscopy.

4.3 Deep Learning for Nanocarrier Localization

While awaiting the availability of DNA origami nanoparticles, we successfully developed the SCP-Nano (Single Cell Precision Nanocarrier Identification) pipeline. This comprehensive system enables single-cell level assessment of nanoparticle biodistribution and mRNA expression in organ tissues and whole mouse bodies.

To demonstrate the versatility of our pipeline, we utilized various nanoparticle types:

1. Rod origami nanoparticles
2. Lipid nanoparticles carrying EGFP mRNA
3. Adeno-associated viruses (AAVs)

The SCP-Nano pipeline incorporates advanced deep-learning algorithms for automated detection and quantification of nanoparticles within complex tissue environments. By leveraging the power of artificial intelligence, we achieved unprecedented sensitivity and accuracy in nanocarrier localization across entire mouse bodies.

Key features of the SCP-Nano deep learning approach:

1. Convolutional neural networks trained on diverse nanoparticle datasets
2. Multi-scale analysis for detection of particles across various size ranges
3. Integration of cellular context information for precise localization
4. Automated quantification of nanoparticle accumulation in different organs and tissues

The development and validation of the SCP-Nano pipeline represent a significant advancement in nanocarrier biodistribution analysis, offering a powerful tool for future vaccine and drug delivery research.

5. Discussion and Implications

The HIVacToGC project has made substantial contributions to nanocarrier-based therapeutics despite the challenges in producing the originally planned DNA origami nanoparticles. The development of the DELiVR and SCP-Nano pipelines represents a significant advancement in our ability to analyze nanocarrier distribution and effects at single-cell resolution throughout the whole organisms.

Key implications of our findings include:

1. **Enhanced sensitivity and precision:** The ability to detect and quantify nanoparticles at extremely low concentrations (0.0005 mg/kg) opens new avenues for studying the biodistribution of therapeutics at clinically relevant doses.
2. **Comprehensive whole-body analysis:** Our pipelines enable the detection of off-target accumulation and potential side effects that may be missed by conventional imaging methods, improving the safety assessment of nanocarrier-based therapies.
3. **Versatility across nanocarrier types:** The successful application of our imaging and analysis techniques to various nanocarrier systems (SiNPs, LNPs, AAVs) demonstrates the broad utility of our approach for diverse therapeutic applications.
4. **Temporal dynamics of nanoparticle distribution:** Our long-term biodistribution studies reveal complex patterns of nanoparticle localization and protein accumulation over time, providing crucial insights for optimizing vaccine delivery strategies.
5. **Potential for germinal center formation:** The observation of concentrated Env-protein areas within lymph nodes suggests the possibility of inducing germinal center formation, a critical step in developing effective vaccines.
6. **Importance of adjuvants:** The differential results observed with or without MPLA adjuvant warn for careful adjuvant selection for nanoparticle-based vaccine design.

These findings have significant implications for developing HIV vaccines and other nanocarrier-based therapeutics. The high-resolution, whole-body imaging capabilities we have developed offer unprecedented opportunities to optimize nanocarrier design, delivery routes, and formulations for enhanced efficacy and safety.

1. Future Directions

While the HIVacToGC project has made substantial progress in developing advanced imaging and analysis techniques for nanocarrier-based therapeutics, several avenues for future research have emerged:

1. **Optimization of DNA origami nanoparticles:** With the imaging pipelines now established, efforts can be refocused on developing and characterizing DNA origami nanoparticles for HIV vaccine delivery.
2. **Refinement of germinal center targeting:** Further studies are needed to optimize nanoparticle design and delivery strategies for efficiently targeting germinal centers within lymph nodes.
3. **Integration of multi-modal imaging:** Combining our high-resolution fluorescence imaging techniques with other modalities (e.g., PET, MRI) could provide complementary information on nanocarrier biodistribution and efficacy.
4. **Expansion to other disease models:** The versatility of our imaging pipelines offers opportunities to study nanocarrier-based therapies for a wide range of diseases beyond HIV.
5. **Artificial intelligence enhancements:** Continued development of deep learning algorithms could further improve nanoparticle detection and quantification speed and accuracy.
6. **Translation to larger animal models:** Adapting our imaging and analysis techniques to larger animal models will be crucial for bridging the gap between preclinical studies and clinical applications.
7. **Investigation of long-term effects:** Extended studies on the long-term fate of nanoparticles and their impact on tissue homeostasis will be important for assessing the safety of nanocarrier-based therapies.

7. Conclusion

The HIVacToGC project has made substantial contributions to the field of nanocarrier-based therapeutics. The development of DELiVR and SCP-Nano pipelines represents a significant advancement in our ability to analyze nanocarrier distribution and effects at single-cell resolution throughout organisms. These tools promise to accelerate the development of precise and safe nanocarrier-based therapeutics by providing comprehensive insights into biodistribution and potential off-target effects at unprecedented resolution and sensitivity. While the project's scope expanded beyond its initial focus on HIV vaccines, the resulting technologies and insights have broad implications for nanomedicine and drug delivery fields. The ability to visualize and quantify nanoparticle distribution with such precision opens new avenues for optimizing therapeutic delivery strategies across a wide range of applications. As we move forward, the foundations laid by this project will continue to drive innovation in nanocarrier design, vaccine development, and drug delivery. The interdisciplinary approach combining advanced imaging techniques, nanotechnology, and artificial intelligence has proven to be a powerful strategy for addressing complex challenges in biomedical research.