J Multidisciplinary Scientific Journal

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Review

Emerging Technologies for the Assessment of Natural Killer Cell Activity

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Abstract: Understanding natural killer (NK) cell functionality is essential in developing more effective immunotherapeutic strategies that can enhance patient outcomes, especially in the context of cancer treatment. This review provides a comprehensive overview of both traditional and novel techniques for evaluating NK cell functionality, focusing on multiparameter assays and spatial methods that illuminate NK cell interactions within their microenvironment. We discuss the significance of standardized assays for assessing NK cell function across various research and clinical settings, including cancer immunotherapy, infectious diseases, and transplantation. Key factors influencing NK cell functionality include the origin of the sample, target–effector ratios, the functional state of NK cells, and the impact of pre-treatment conditions and their natural aging effect on NK cell activity. By emphasizing the importance of selecting a suitable technique for reliable measurements, especially for longitudinal monitoring, this review aims to give an overview on techniques to measure NK cell functionality in vitro and show the interaction with their microenvironment cells by spatial imaging. Ultimately, our understanding of NK cell functionality could be critical to biomarker development, drug design, and understanding of disease progression in the field of oncology or infectious disease.

Keywords: NK cell functionality; cytokine release; standardized assays; multiparameter analysis; spatial techniques

Citation: Grab, A.L.; Nesterov-Müller, A. Emerging Technologies for the Assessment of Natural Killer Cell Activity. *J* **2024**, *7*, 457–471. https://doi.org/10.3390/j7040027

Academic Editor: Pietro Cipresso

Received: 31 July 2024
Revised: 27 September 2024
Accepted: 23 October 2024
Published: 7 November 2024

1. Introduction

NK cells are key components of the innate immune system and play a crucial role in defending against cancer and various diseases [1–3]. The cytotoxic efficiency of NK cells is determined not only by their differentiation state [4] but also by their ability to form immunological synapses [5,6] and release cytotoxic granules containing perforin and granzymes [7]. Recent studies have identified different functional states of tumor-infiltrating NK cells, such as the ratio of dysfunctional stressed and cytotoxic subsets, that correlate with patient survival across multiple cancer types, highlighting the importance of understanding these states in therapeutic applications [2]. This multifaceted role underscores the potential of NK-based therapies, including cell-based therapies, antibodies, and CAR-NK (Chimeric Antigen Receptor natural killer) cells, in targeting a wide range of malignancies and viral infections [1,3]. The latest advancements in NK cell-based therapies [8] have generated significant interest in the field of oncology. The investigation of cell phenotypes in the tumor microenvironments has shown exhaustion and immunosuppressive metabolites [9]. Understanding the distribution and function of cellular subsets in bone marrow, lymphatic systems, and peripheral blood is crucial in optimizing their therapeutic potential [10]. NK cell therapies are increasingly being developed in conjunction with both antibodies [11] and cell-based therapies, leveraging the unique mechanisms of action of

each approach to enhance the anti-tumor efficacy. While allogenic and autologous NK cell therapies are safe and widely used in clinical therapy, they can show low efficiency due to the suppression of Major Histocompatibility Complex or a lack of target molecules [12]. Monoclonal antibodies mediate the process through which NK cells recognize tumor cells, form a synapse, are engaged through antibody-dependent cellular cytotoxicity, and kill tumor cells [13]. This mechanism has been effectively utilized in therapies targeting specific antigens, such as CD20 in B-cell malignancies [13,14]. The development of NK cell engagers has demonstrated exciting potential in treating hematological malignancies. For instance, Sanofi's CD16-NKp46-CD123 trispecific antibody has shown remarkable efficacy against acute myeloid leukemia (AML), leading to FDA (Food and Drug Administration) fast-track approval [15]. On the other hand, cell-based therapies, including CAR-NK cells [16], involve engineering NK cells to express chimeric antigen receptors that allow for the direct targeting of tumor antigens independent of MHC restriction [16,17], leading to improved patient outcomes [18,19]. Genetic engineering techniques have further enhanced NK cell functionality. CRISPR/Cas9 technology offers the ability to knock out inhibitory receptors, thereby boosting NK cell activity against tumors [20].

However, the functionality of NK cells is closely linked to their metabolic regulation [21,22]. NK cell activation is connected to metabolic pathways that provide the necessary energy and biosynthetic precursors for their effector functions, including proliferation, cytokine production, and cytotoxic activity [23].

To fully comprehend NK cell mechanisms of action and their interactions with the microenvironment, advanced techniques are essential. While the chromium release assay was long considered the gold standard for assessing NK cell cytotoxicity, flow cytometry now allows for the investigation of microenvironmental markers [24] and more detailed functional analysis [25]. Customized assays enable cytokine release analysis, and companies offer NK panels for cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) and gene sequencing, providing unprecedented insights into NK cell biology at the single-cell level [26].

Spatial imaging techniques have emerged as powerful tools for understanding NK cell distribution, invasion in tumor tissues, and cellular cross talk. Methods such as those provided by spatial technologies [27] allow us to identify and visualize the relationships between different cells, next-neighbor analysis, and local distribution, which is critical in elucidating NK cell interactions within the tumor microenvironment [28]. Applying these techniques can be critical in finding novel biomarkers and improving prediction or evolving parameters for cellular behavior and in designing the experimental setup (Figure 1).

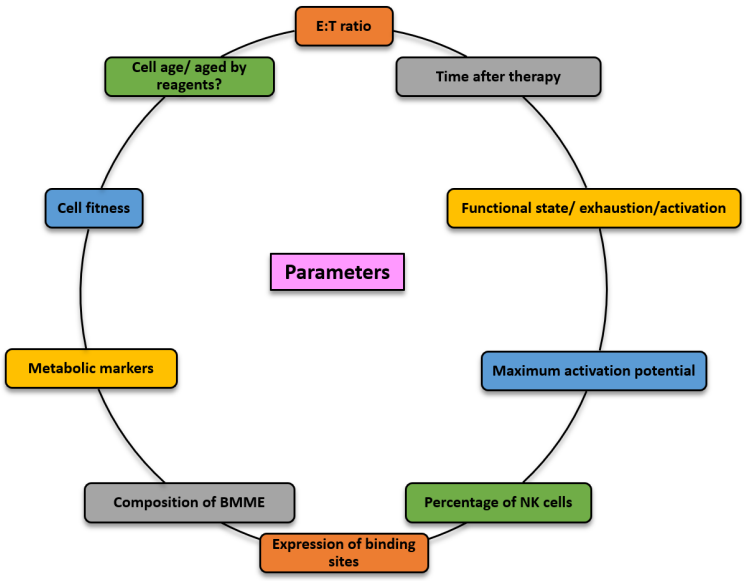

Figure 1. Ten key parameters to be considered in NK cell co-culture experiments.

This review aims to provide a comprehensive summary of both traditional and novel methods for elucidating NK cell function and metabolic regulation. We will discuss the advantages and limitations of these techniques, offering insights into the rapidly evolving field of NK cell-based cancer immunotherapy. This will be of major interdisciplinary interest for a broad readership in the field of oncology, immunological diseases, and engineers and allow us to monitor NK cells' functional state, metabolism [29], and interactions with their microenvironment. This could help us to select the optimum assay and could potentially lead to the identification of targetable disease mechanisms.

2. Traditional Methods Like Chromium Release Assays Assess NK Cell Function

Over recent decades, chromium release assays have been the gold standard for determining the cytotoxicity of NK cells, particularly in the context of cancer research. This assay involves labeling target cells with radioactive Cr-51 [30,31]. During cell lysis, the cell membrane becomes damaged and Cr-51 is released. This assay determines the percentage of lysed cells by the amount of chromium released into the supernatant as an indicator of NK cell function [30,31]. This method allows researchers to quantify NK cell activity in a controlled environment and study the effectiveness of compounds [29]. For example, this assay has been used to investigate the effects of therapeutic agents, such as Rituximab, on NK cell function against Raji cells, demonstrating how external stimuli can enhance or inhibit immune responses [30,31].

Despite its widespread use, the chromium release assay has limitations, including the requirement for radioactive materials and the inability to measure cytotoxicity at multiple time points. Researchers have investigated protocols to measure cell viability, measure cellular metabolic activity, and NK cell proliferation by replacing radioactive compounds, by flow cytometry [32] or plate reader approaches using calorimetric, bioluminescent, or fluorescent live/dead assays, e.g., the WST-1 assay [33], CellTiter-Glo [32], MTT [34], or Calcein release assays [35]. Consequently, we present here advanced methodologies that address the limitations of the 51Cr release assay while allowing the measurement of time-resolved dynamics and additional parameters.

3. Novel Approaches to the Detection of NK Cell Function

Innovative methods for assessing NK cell function, such as image-based flow cytometry, impedance-based real-time cell analysis systems, microfluidic platforms for single-cell analysis, bioluminescence imaging techniques, and electrochemical biosensors for cytokine detection, present significant advantages over traditional techniques. These novel approaches will be addressed in the following paragraphs.

3.1. Flow Cytometry as Tool to Characterize NK Cell Function and Metabolic State

Flow cytometry has become an essential tool for evaluating the expression patterns of multiple inhibitory and stimulatory receptors on NK cells [36]. Cossarizza et al. have published guidelines on the tricks and pitfalls to avoid when dealing with immunological samples in flow cytometry [37]. This technique allows researchers to resolve donor-specific differences in NK cell heterogeneity and simultaneously assess the expression of receptors and epitope integrity using advanced multicolor flow cytometry panels. One significant advantage of flow cytometry in clinical studies is its ability to perform high-dimensional analysis of stimulatory and inhibitory markers on NK cells [38,39].

This capability is crucial in assessing the functional status of NK cells and understanding their role in immune responses [40]. However, the accuracy of flow cytometry can be affected by cell isolation steps; e.g., tissue digestion might downregulate epitope expression, and the clone of the antibody for targeting specific surface markers might be critical to successful staining [41]. Furthermore, dynamic ranges are difficult to access [38].

To address these limitations, modern techniques such as Met-Flow have been developed [38]. Met-Flow is a flow cytometry-based method that enables single-cell metabolic analysis by targeting key proteins and rate-limiting enzymes across multiple pathways.

This approach captures the metabolic state of immune cells, providing insights into the complex interaction of the anabolic and catabolic metabolism that underpins the ability of leukocytes to mount an immune response [38]. Metabolic reprogramming [42–44] is crucial in the effector function of NK cells [45], and Met-Flow allows researchers to interrogate this network of metabolic pathways at the single-cell level within a heterogeneous population [38].

Moreover, image-based flow cytometry [46] analysis and sorting represent a significant advancement in the field. This technology not only allows for the detailed analysis of cells but also enables sorting based on specific characteristics observed in the images. For instance, the BD CellView™ Image Technology combines high-speed imaging with traditional flow cytometry [46], capturing multiple images of individual cells at a rate of 15,000 cells per second. This allows for sorting based on detailed microscopic image analysis, providing a more comprehensive understanding of cell functionality. However, other studies report on the impact of sorting procedures on NK cell metabolism, introducing oxidative stress and changing metabolite concentrations, which cannot be fully mitigated by buffer additives or data normalization techniques [47].

3.2. Impedance-Based Real-Time Cell Analysis Systems for Label-Free Monitoring of Interactions Between NK Cells and Target Cells

Impedance-based systems facilitate the kinetic analysis of cytotoxicity and capture dynamic interactions over time [48,49]. This capability allows us to resolve temporal aspects of NK cell function that are often overlooked in single-point assessments. The absence of labeling requirements in impedance-based systems further enhances their utility. Impedance-based systems provide valuable kinetic and mechanistic insights into NK cell cytotoxicity [49]. The continuous monitoring capabilities of impedance-based systems also help to minimize inter- and intra-assay variability, leading to more consistent and reproducible results [48].

3.3. Cutting-Edge Microfluidic Approaches to the Analysis of Individual NK Cell–Target Cell Interactions

High-resolution microfluidic methodologies have transformed the study of NK cell heterogeneity and functionality [50], addressing the limitations of traditional bulk assays like chromium release assays and flow cytometry [50]. These advanced platforms enable researchers to identify rare NK cell subsets often masked in bulk analyses, providing a deeper understanding of NK cell diversity. Their ability to handle limited clinical samples is particularly valuable in scenarios where sample availability is constrained, offering both spatial and temporal resolution that traditional methods cannot achieve [51]. Recent advancements in microfluidic technology have driven the development of lab-on-chip devices, including microfluidic sensors and paper-based chip sensors [50,52]. Automated droplet-based platforms [53] capable of the real-time monitoring of NK cell interactions with target cells [54] and multiplexed cell-based assays allow apoptosis assays and dose–response assays [55,56]. For instance, one study screened over 60,000 droplets, identifying approximately 2000 individual cellular interactions over a 10 h period [50]. It found that around 20% of NK cells exhibited positive cytotoxicity against K562 cells, with most activity occurring within the first four hours. This highlights the heterogeneity within the NK cell population, revealing that individual cells have varying strengths in their effector functions [50]. Furthermore, these platforms facilitate the integration of advanced sequencing and barcoding technologies into high-throughput applications [57] for immunological research, which could be used for the identification and characterization of functional NK cell subpopulations. One drawback to this technique is that the cells are taken out of their microenvironment and specific culturing processes, such as patient-derived organoids [58] or patient-derived xenografts, which complicate longitudinal studies [59].

3.4. Bioluminescence Cell Tracking and PET Techniques Enable Real-Time Monitoring of NK Cell Activity

Particularly suitable techniques for longitudinal studies include FACS assays [60] or the monitoring of longitudinal transcriptional changes [61]. However, for tracking cell migrations, often, bioluminescence imaging assays [62] are used, allowing researchers to investigate NK cell dynamics over extended periods [63]. One of the significant benefits of bioluminescence imaging is its ability to provide spatial information about NK cell localization and function within the tumor microenvironment [63]. For instance, studies have demonstrated that NK cells can be visualized as they infiltrate tumors, offering insights into their interactions with cancer cells in real time [64]. NK cell metabolic profiles are influenced by exposure to the immunosuppressive conditions of the tumor microenvironment, which can significantly impact their functionality and cytotoxic capabilities. Bioluminescence imaging enables us to perform in vivo monitoring [65], which also allows researchers to assess NK cell function in a more physiological context [62], providing a clearer picture of how these cells operate in their microenvironment. Additionally, recent advancements in imaging technologies, particularly positron emission tomography (PET), have significantly improved our ability to visualize and track NK cell activity within living organisms [66]. This is particularly relevant in studying the effects of various cytokines on NK cell metabolism and activity. For example, a recent study utilized PET imaging to track NK cells labeled with Zr89-oxine for up to two weeks [67]. The results demonstrated an initial accumulation of NK cells in the lungs, followed by redistribution to the liver and spleen, consistent with the expected biodistribution of immune cells post-intravenous injection [67,68]. Notably, the ability to visualize NK cell localization and function in real time enhances our understanding of their metabolic demands and functional states during these processes, as metabolic reprogramming is crucial in NK cell activation and effector functions, including cytotoxicity and cytokine production [68].

3.5. Advanced Multiplexed Imaging Systems for NK Cell Functionality and Metabolism

Multiplexed imaging techniques [69–73] are used to investigate spatial information [74] with transcriptomic profiles [75], allowing researchers to explore how NK cells interact with their surroundings [76] in real time and thereby providing critical insights into their roles in immune responses and therapeutic efficacy [77]. A study using multiplexed immunofluorescence identified that NK cells (NCAM1+CD3−) are rare in colorectal cancer but show high granzyme B expression and proximity to tumor cells, correlating with longer cancer-specific survival, emphasizing the importance of spatial context in the tumor microenvironment [77]. Lunaphore's high-resolution spatial transcriptomics platforms enable the mapping of NK cell distributions and their functional states within tissues [78]. This capability allows researchers to assess the spatial organization of NK cells in relation to tumor architecture, which is crucial in understanding their metabolic adaptations and functional dynamics in the tumor microenvironment [78,79]. Other studies have utilized CODEX [80], a method of tagging antibodies with oligonucleotides, enabling high-parameter imaging through iterative hybridization and dehybridization cycles. This allows researchers to visualize complex cellular interactions and spatial organization effectively [81] or use Nanostring's GeoMx Digital Spatial Profiler for analyzing NK cell functionality by quantifying gene expression in specific tissue regions [82]. Information on the transcriptional profiles of NK cells under different conditions can help us to understand their roles in tumor immunity. For example, studies have shown that the metabolic reprogramming of NK cells [42], characterized by shifts from oxidative phosphorylation to glycolysis, can be directly correlated with their cytotoxic capabilities, revealing how NK cells adapt their metabolism in response to the tumor microenvironment [77].

Another well-established technique is live-cell imaging with the IncuCyte system (Sanatorius GmbH, Libušina, Czechia), which enables the continuous real-time monitoring and quantification of cellular events such as apoptosis and proliferation directly within a standard cell culture incubator [83]. This is particularly useful in assessing NK cell activity [84] in various tumor models, including 3D spheroids [85,86], where it can track

changes in cell viability and apoptosis through fluorescent markers [87]. The ability to perform longitudinal analysis [86] enables researchers to observe the dynamics of NK cell-mediated killing and infiltration within the tumor microenvironment [88,89]. However, the high cost of the IncuCyte system and its limitations in suspension cultures must be considered when designing experiments. In light of these challenges, recent advancements in high-dimensional single-cell analysis offer promising alternatives for assessing NK cell functionality [88,89].

3.6. High-Throughput Screening Platforms for NK Cell Functional Assessment

Recent advancements in high-dimensional single-cell analysis, particularly single-cell RNA sequencing and cellular indexing of transcriptomes and epitopes by sequencing (CITE-seq) [90], have significantly enhanced our understanding of NK cell heterogeneity [76]. These technologies have revealed distinct NK cell subsets with unique functional roles, involvement in cytokine production (CD56 bright) or potent cytotoxic capabilities (CD56 dim). However, enhancing NK cell functionality remains a challenge [91]. Exploiting the cytotoxic efficacy of NK cells through therapeutic strategies is of great interest in understanding novel cancer therapies, such as adoptive NK cell therapy and bispecific killer engagers [8,15]. Strategies to augment NK cell activity include modulating signal transduction pathways, manipulating inhibitory and activating receptors, and employing cytokine-induced activation [68]. Various aspects of NK cell biology can be shown by ATAC-seq, which requires only 50,000 to 100,000 cells [92]. ATAC-seq can help to identify open chromatin regions across the NK cell genome, which are associated with active gene expression and elucidate the mechanism of NK cell differentiation and function [93]. For example, ATAC-seq has revealed the differential expression of effector molecules like perforin and granzyme B during NK cell activation, which correlates with their cytotoxic potential against various tumor types [94]. Additionally, this technique was utilized to identify transcription factor binding sites in NK cells, providing insights into the key regulators of their activation and function [95].

These techniques deliver huge data sets to quantify NK cell interactions and activities in various experimental setups with the incorporation of machine learning algorithms [96]. These models can distinguish between cancerous and non-cancerous samples with high accuracy, utilizing NK cell activity as a predictive biomarker for cancer diagnosis or treatment response.

3.7. Murine Models for Studying NK Cell Biology and Function

Mice can serve as model organisms [97] to provide a controlled environment for studying NK cell interactions and mechanisms [98] with defined genetic modifications [99]. The evaluation of NK cell functionality using immunocompetent, immunodeficient, and humanized murine models is essential in understanding NK cell therapies. Humanized models like Human Immune System mouse models [97] are genetically modified mice that have been engrafted with human cells or tissues to study aspects of human immunobiology and diseases. These models are particularly useful for studying human-specific pathogens and evaluating immunotherapies. MISTRG mouse models incorporate specific human cytokines to enhance human immune cell differentiation from progenitor cells and NK cell functionality. For immunotherapy tests on activating receptor NKP46, humanized NKP46 mouse models have been generated by the knock-in of human NKP46 alleles [100]. The model allows for extended observation periods, enabling researchers to study the dynamics of NK cell responses over time [100].

Immunodeficient mouse models like NSG [101] and NOG [102] have been widely used to study NK cell functionality due to their ability to support the engraftment of human cells and tissues [103]. These models lack T, B, and NK cells, providing a highly immunodeficient environment conducive to studying human NK cell responses in vivo [104]. Immunocompetent mouse models, such as syngeneic and transgenic models [105], have the ability to support the engraftment of human cells, enabling the study of human NK cell

biology and functionality in vivo [106]. Immunocompetent models [105], on the other hand, provide a more physiologically relevant system to study NK cell interactions with tumors and test NK cell-based therapies, as they possess a fully functional immune system [105]. Immunocompetent mouse models have a fully functional immune system, allowing them to mount an immune response similar to that of humans [104]. These models can be utilized to observe NK cell interactions with other immune cells and the tumor microenvironment [107,108]. Syngeneic mouse models, which involve transplanting tumor cells from the same genetic background into immunocompetent mice, are useful for studying NK cell-mediated tumor growth inhibition [109]. Transgenic mouse models [110], genetically modified to express specific human genes or oncogenes, enable the investigation of NK cell functionality in particular diseases, such as the study of NK cell interactions with glioblastoma in C57BL/6 mice [111,112].

4. Discussion

In this study, we gave an overview on techniques for measuring the functionality and activity of NK cells (Figure 2), which is essential in the context of immunotherapies, particularly as these therapies have shown promising outcomes in clinical trials [113].

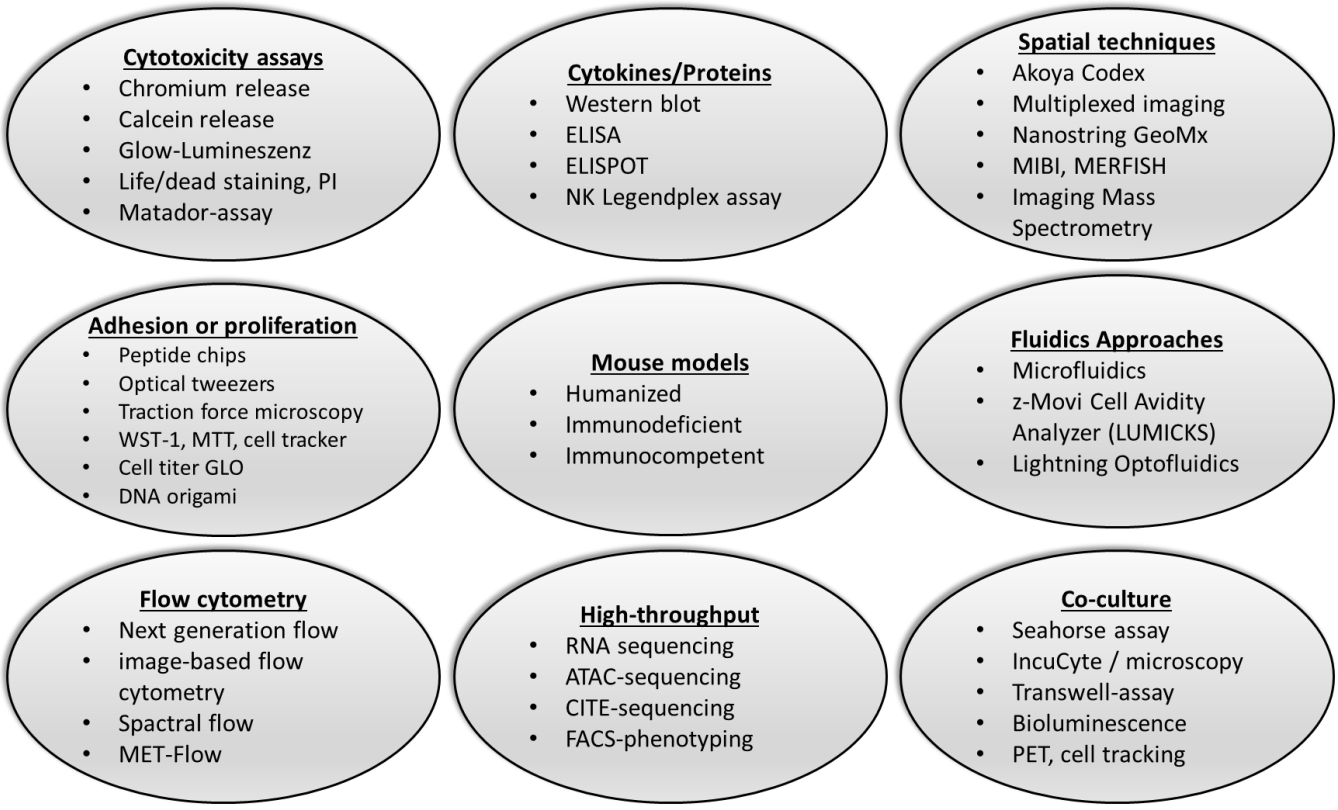

Figure 2. Overview of Techniques for Assessing NK Cell Functionality.

Standard methods allow us to obtain different information based on the experimental design and expected readouts. Traditional methods such as the chromium-51 release assay provide valuable insights into NK cell functionality but face limitations in capturing the full complexity of NK cell biology and function (Table 1).

It is often crucial that we combine techniques in order to examine multiple aspects, and for data interpretation, it is important to look at cellular behavior, cellular differentiation state, functionality, phenotype, and metabolism. The latter can be investigated with the Agilent Seahorse XF assay for the real-time assessment of NK cell metabolism [114], which was not discussed here. This assay requires a huge NK cell amount (e.g., 100,000 cells per condition) [115], which can be a limiting factor in some experimental settings.

Thus, researchers have focused on integrating metabolic markers into their FACS setup or used mass spectrometry and single-cell metabolomics [116]. These techniques have recently gained significant attention due to new avenues for NK cell engineering, which modulates NK cell functionality and directs them to specific targets. Engineering NK cells with chimeric antigen receptors (CARs) [117] enhances their ability to target specific tumor antigens [115], allowing for the precise tracking of their efficacy in real time. For instance, metabolic reprogramming to favor glycolysis over oxidative phosphorylation has been linked to increased cytokine production and target cell lysis. Understanding these metabolic shifts is key to ensuring that NK cells remain active and competent in challenging environments [115]. The combination of advanced techniques with traditional methods offers a more comprehensive understanding of NK cells and their mechanism of action. One important point in this context is the imaging of NK cells in vivo. Non-invasive imaging technologies, such as positron emission tomography (PET), have emerged as powerful tools for assessing NK cell function in vivo. By labeling NK cells with radiotracers, researchers can visualize their migration to tumor sites and evaluate their functional status, providing insights into therapeutic efficacy [67]. While this approach offers unprecedented in vivo monitoring capabilities, it also raises concerns about the potential impact of radiolabeling on NK cell function and the limited resolution for detecting individual cell interactions. Researchers must carefully consider the strengths and limitations of each approach to draw accurate conclusions about NK cell functionality.

Table 1. Overview of Techniques for Assessing NK Cell Function.

Technique	Required Cell Count	Cell Isolation	Staining Technique	Kinetics	Long-Term Culture	Interaction with Microenvironment	Ref.
Chromium release assay	+++	Yes	Radioactive labeling	Limited	No	No	[30,31]
FACS	++	Yes	Fluorescent antibodies	Good	No	Limited	[32,40]
Impedance monitoring	+	Yes	None	Excellent	Yes	Limited	[48]
Microfluidics	+	Yes	Varies	Excellent	Limited	Yes	[50–54]
Bioluminescence imaging	++	Yes	Bioluminescent lable	Good	Limited	Yes	[62]
PET	+++	No	Radiotracker	Good	No	Yes	[66,67]
Multiplexed imaging	++	No	Antibodies	Limited	No	Yes	[70]
Lunaphore	++	No	Antibodies	Limited	No	Yes	[68,69]
Codex akpya	++	No	Antibodies + Dyes	Limited	No	Yes	[70]
Geo Mx Profiler	+	No	Antibodies	Limited	No	Yes	[72,73]
Cite-seq	+	Yes	Antibody-oligo conjugates	Limited	No	Limited	[90]
RNA-seq	+	Yes	None	Limited	No	Limited	[90]

+ indicates the number of cells, + Low cell count, ++ 10,000–100,000 cells, +++ are more than 100,000 cells.

5. Conclusions

Advanced technologies, standardized control samples, and gold-standard methods are crucial in improving NK cell-based immunotherapies. The combination of traditional

assays with cutting-edge technologies provides a more comprehensive view of NK cell functionality, enabling researchers and clinicians to optimize immunotherapies and monitor their efficacy. As NK cell-based therapies continue to evolve, the development of standardized, multi-modal approaches for assessing NK cell function will be essential in advancing our understanding of NK cell behavior.

6. Future Directions

We hypothesize that, when developing new techniques, we should aim for simultaneously assessment based on metabolic parameters and functional readouts [29], since combining metabolic profiling with traditional functional assays could provide a more comprehensive understanding of NK cell [38] behavior during clinical trials. One option comprises high-resolution imaging techniques and single-cell analysis platforms that provide a greater view of NK cell heterogeneity and function within complex tissue microenvironments [117]. These approaches may reveal new functional subsets of NK cells [118] and identify novel targets for therapeutic manipulation.

High-density peptide arrays have been used to study the interactions of short peptides with cells [118–120], RNA [121], or proteins [122], as well as to analyze the effects of peptides on cell adhesion or repulsion at the individual amino acid level [123]. This technology has been successful in identifying selective peptides that either strongly attract or repel cancer cells, making it suitable for further application in NK cell functional assessment.

High-density peptide arrays can be used to screen for peptides that enhance NK cell adhesion and activation on cancer cells, potentially improving the presentation of tumor antigens and increasing the visibility of cancer cells to NK cells for targeted destruction. By using diverse peptide libraries, it is possible to discover sequences that modify cell surface receptors or ligands, thereby enhancing NK cell recognition and killing efficiency [123,124].

Additionally, repellent peptides could be combined with NK cell-activating peptides to create surfaces that isolate cancer cells and make them more susceptible to NK cell-mediated killing [125]. This technology can be integrated into microfluidic systems for studying NK cell and cancer cell interactions under controlled conditions [126]. It also enables the discovery of new ligands for NK cell receptors, which can be tested to determine their effect on NK cell activation [118–120].

Regarding experimental setups, cancer cells can first be cultured on peptide arrays to determine which peptides promote or inhibit their adhesion and growth. Afterward, NK cells can be introduced to observe how the presence of specific peptides influences NK cell recognition and cytotoxicity against cancer cells. The activation of NK cells can be monitored by measuring the release of Granzyme B or Perforin [127], the production of IFN-y [128], or changes in activation markers like CD69 and CD107a [128].

Thus, the main advantages of using high-density peptide arrays include high throughput and efficiency, as they allow the simultaneous testing of many peptide sequences, and rapid screening, enabling the quick identification of peptides that either enhance NK cell activity or induce resistance in cancer cells [129]. These findings pave the way for the further exploration of high-density peptide arrays in current clinical directions for cancer immunotherapy. Current clinical directions show a shift towards NK cell-based therapy approaches [130] as reasonable alternative [118,131,132], when T cell-based therapies face limitations, e.g., by T cell depletion or exhaustion [133,134]. Future clinical trials are likely to explore combination therapies that help to overcome drug resistance and improve overall response rates.

Author Contributions: Conceptualization, A.L.G.; writing—review and editing, A.L.G. and A.N.-M., visualization, A.L.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the conclusions of this article will be made available by the authors on request.

Acknowledgments: All authors thank the members of Clinical Cooperation Unit Molecular Hematology/Oncology, DKFZ, and Department of Medicine V, Medical Faculty Heidelberg and University Hospital, Heidelberg University, for their great collaboration. The authors acknowledge the use of AI-based tools (Perplexity and ChatGPT) for providing information and resources that contributed to the preparation of the manuscript, as well as for language editing and assistance in improving the clarity of the text.

Conflicts of Interest: The authors declare no conflicts of interest.

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