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Viewpoint

Label-Free Dynamic Mass Redistribution Assay To Characterize Holistic Chemokine Receptor Pharmacology in Neutrophils

In a study published in this issue of JPET, Stott et al. (2024) investigated the capacity of the label-free dynamic mass redistribution (DMR) assay to accurately characterize chemokine receptor pharmacology in primary neutrophils and the extent to which this approach offers advantages over traditional high-throughput screening methodologies. Screening for molecules to modulate activity of a target protein is a vital step of the modern drug discovery process. Initial screening campaigns usually involve testing thousands to millions of drug-like molecules for desired effects in high-throughput assays (Addis et al., 2023). Such assays are commonly based on cellular approaches where cell lines overexpress recombinant target proteins such as G protein–coupled receptors (GPCRs), tyrosine kinase receptors, ion channels, transporters, enzymes, etc. Alternatively, some high-throughput assays are conducted directly on purified target proteins. In cell-based assays, activation of the target protein leads to recruitment of effector proteins and initiation of downstream signaling events, which can be measured using a variety of techniques. Most of these detection techniques require some form of labeling, either of the target protein itself or the detection reagents/biosensors. For example, interactions between the target and effector proteins can be followed by proximity assays [i.e., split enzyme complementation, bioluminescence resonance energy transfer (BRET), and fluorescence resonance energy transfer (FRET)] where each protein is labeled in such a way that a specific signal is generated when the two proteins associate. Essential for all high-throughput assays is a high signal-to-noise ratio and the ability to be miniaturized to a format that allows thousands of molecules to be tested at the same time (Addis et al., 2023).

In most high-throughput screening approaches, the target protein exists under conditions that differ significantly from its native environment. These nonphysiologic conditions include exogenous overexpression of the target protein far beyond endogenous levels, direct labeling of target/effector proteins, and/or complete isolation of the target protein from a cellular environment (in purified protein-based assays). Although these modifications are often tolerated in the high-throughput assay format, they sometimes influence the pharmacological properties of the target protein and downstream events, potentially leading to artifactual results (Kenakin, 2004, 2017). Therefore, 'hit' molecules identified in high-throughput screening assays cannot always be translated directly into physiologic systems, which somewhat limits the usefulness of this type of screening approach.

One strategy for addressing the challenges associated with traditional high-throughput approaches is to perform the screening using primary cells that are physiologically relevant and express the target protein endogenously. Not only does the target exist in its native environment, but the usage of primary cells allows identification of pharmacological molecules that modulate desired target-stimulated physiologic functions using mechanism-based assays. Examples of physiologic functions that can be measured include hormone secretion, cellular proliferation, regulation of disease-associated biomarkers, chemotaxis, etc. Although a mechanism-based screening approach logically appears to be superior in identifying hit molecules with translational potential to traditional high-throughput assays, these approaches come with their own drawbacks. For example, most mechanism-based assays are low throughput, labor intensive, prone to assay variability, and/or highly susceptible to interdonor variability (Al-Ali et al., 2004; Lamore et al., 2004; Gomez-Lopez et al., 2011; Silvestre-Roig et al., 2019). These drawbacks make them unsuitable for the intensive screening campaigns that are typically performed in the industry.

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ABBREVIATIONS: BRET, bioluminescence resonance energy transfer; DMR, dynamic mass redistribution; GPCR, G protein–coupled receptor; FRET, fluorescence resonance energy transfer.

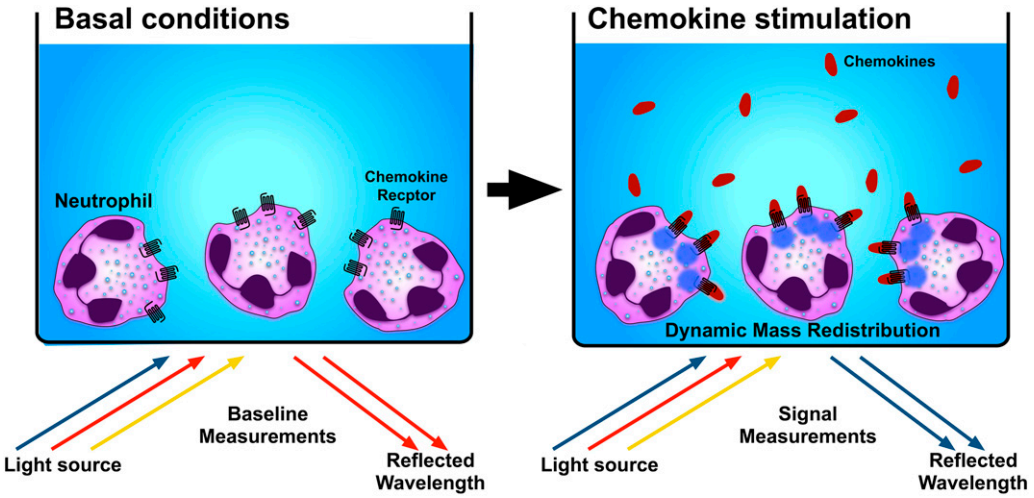

Fig. 1. Label-free dynamic mass redistribution (DMR) detection of chemokine-stimulated neutrophils.

A relatively new method that promises to overcome issues with traditional high-throughput assays is label-free DMR assays. The DMR approach is based on two alternative detection methods, including light refraction and electrical impedance (Scott and Peters, 2010). The light-based method utilizes optical biosensors to detect alterations in the refractive index of cells arising from signaling, protein trafficking, rearrangement of cytoskeleton and adhesion, or morphologic changes (Fig. 1). In the impedance-based method, these cellular activities are measured as a change in electrical impedance of current flow between two electrodes. A unique feature of the DMR approach is that the experimental readout is not directly derived from a specific molecular event within the cell but represents a more integrated and 'holistic' response to cellular stimulation. Furthermore, the detection of cellular responses is conducted in a completely label-free manner and is thus minimally influenced by the experimental conditions. Although label-free DMR assays hold great promise in studying pharmacology and cellular signaling in a more native and accurate manner, the technique is still relatively new and not broadly validated.

In the Stott et al. (2024) study, the investigators applied label-free DMR assay to characterize the chemokine receptors CXCR1 and CXCR2 in primary neutrophils where they are endogenously expressed and regulate cell migration (Metzemaekers et al., 2020). As the most abundant leukocyte in circulation and a key regulator in disease processes, neutrophils are becoming important to target pharmacologically (Németh et al., 2020). To initiate the study, a range of CXCR1/2-selective chemokine ligands and antagonists were tested in CHO cells overexpressing either CXCR1 or CXCR2. Responses were quantified by both $G_{i/o}$ protein activity (inhibition of forskolin-stimulated cAMP production) and label-free DMR assays. In general, there was reasonable agreement in the pharmacology of both receptors between the two assays. However, although most chemokines acted as full agonists in the $G_{i/o}$ protein activity assay, some of them only behaved as partial agonists or had reduced potencies in the DMR assay. This discrepancy between the $G_{i/o}$ protein activity and DMR assays was speculated to be due to signal amplification in the $G_{i/o}$ protein assay. However, this seems unlikely, as the chemokine-stimulated holistic response measured by the DMR assay integrates signal amplification that is generated throughout the signaling cascade. Thus, the underlying reason for these differences is not obvious.

Next, the authors characterized the chemokines and antagonists in primary neutrophils isolated from human donors. Using the label-free DMR assay, the chemokines and antagonists tested displayed varying degrees of correlation with the pharmacological data obtained from the recombinant CHO system where CXCR1 or CXCR2 was overexpressed. For example, an excellent correlation in chemokine potencies was observed between the DMR assay in isolated neutrophils and the $G_{i/o}$ protein activity assay in CHO-CXCR2 cells (Fig. 2). However, the correlation in chemokine potencies was less impressive between the DMR assays in isolated neutrophils and CHO-CXCR2 cells. The reason behind the sometimes suboptimal correlations between the different assays across the two cell systems was not elucidated in the study, and characterization of $G_{i/o}$ signaling in neutrophil was also not performed due to technical limitations. Interestingly, the response to chemokine stimulation in isolated neutrophils was much larger than CXCR2-overexpressing CHO cells, suggesting that the native system differs significantly from the engineered system, which might explain why the two systems are difficult and/or inappropriate to compare. Despite these differences, the CXCR2-mediated DMR responses in isolated neutrophils were blunted by CXCR2 antagonist in a manner very similar to the $G_{i/o}$ protein activity assay in CHO-CXCR2 cells.

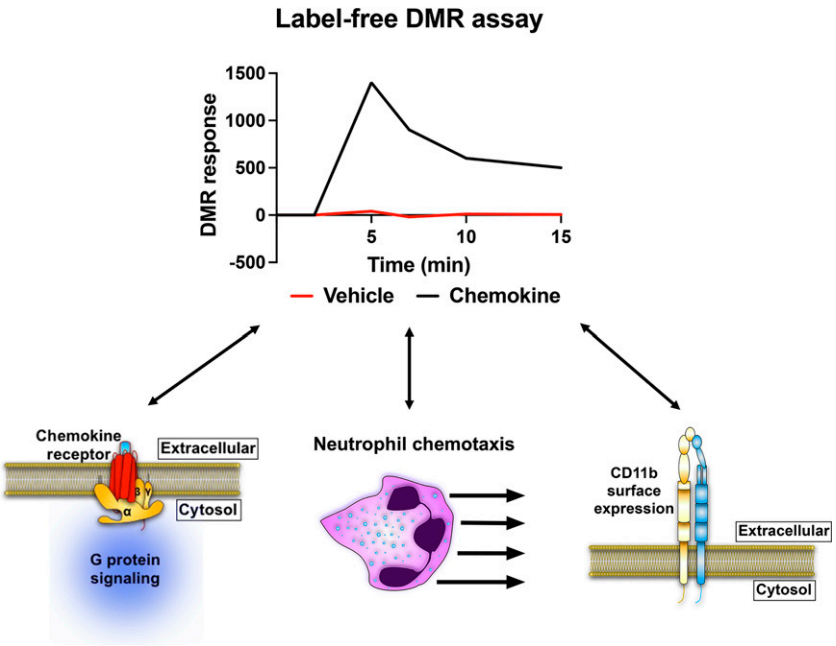

Fig. 2. The dynamic mass redistribution (DMR) response to chemokine receptor stimulation correlates with G protein signaling, chemotaxis, and surface expression of CD11b.

To further validate the label-free DMR approach in isolated neutrophils, chemokine-induced DMR responses were compared with cell biologic/physiologic outputs. These outputs include in vitro chemotaxis and enhanced surface expression of the integrin CD11b, a validated CXCR2 engagement biomarker in neutrophils (Lazaar et al., 2011; Miller et al., 2015). Overall, chemokine stimulation of neutrophils in these physiologic-relevant assays displayed similar capacity to promote chemotaxis and upregulation of CD11b as cellular activation in the DMR assay (Fig. 2). However, the variability between different donors of neutrophils was much higher in the physiologic-relevant assays compared with the DMR assay.

Finally, neutrophils express other GPCRs, including formyl peptide receptors, leukotriene B4 receptors, platelet activating factor receptor, complement component 5a receptor 1, free fatty acid receptor 2, and purinergic P2Y receptors. Some of these GPCRs couple to a different profile of G protein subtypes compared with CXCR1/2, and thus might not yield the same type of integrated response in label-free DMR assays as these chemokine receptors. Therefore, the investigators tested how stimulation of each of these receptor families promotes changes in the DMR response. All ligands tested elicited DMR responses in neutrophils that were shown to be reproducible and robust over multiple donors. All ligands resulted in a rapid initial peak within the first few minutes of stimulation, similar to that observed with the CXCR1/2 chemokines, and for some receptors a second peak at a later time point (leukotriene B4 receptors). The magnitude of the responses varied from receptor to receptor, which could potentially be a result of variation in receptor expression, downstream signaling profile, and/or cell physiologic responses. However, the reasons behind these differences were not investigated experimentally in this study. Overall, these observations agreed with previously reported data by other laboratories, which highlights a decent reproducibility of the label-free DMR assay (Christensen et al., 2017).

In the industry, there is a growing recognition of the significance of primary cell screening in enhancing the translation of new pharmacological entities to clinical applications. To this end, the label-free DMR assay is a new and interesting approach that allows medium-throughput screening in primary cells using a relatively simple experimental setup. In neutrophils, an excellent correlation between DMR responses and physiologic outputs such as chemotaxis and surface expression of CD11b was demonstrated in the current study (Fig. 2). In addition, examples of unique pharmacological features specific to neutrophils, but not observed in CXCR1- or CXCR2-overexpressing CHO cells, were found using the DMR assay. Thus, the label-free DMR approach appears to have some advantages over traditional high-throughput assays, labor-intensive screening approaches in primary cells such as high content imaging, and/or low-throughput mechanism-based assays. Several studies, including the current work, have studied the pharmacology of GPCR in neutrophils (Schröder et al., 2011; Locker et al., 2015; Christensen et al., 2017; Frei et al., 2021), but label-free DMR assays have also been applied to characterize GPCR pharmacology and additional pharmacologically relevant processes in other primary cells, including osteoblasts, endothelial cells,

neurons, keratinocytes, and stem cells (Schröder et al., 2010; Pai et al., 2012; Piccinno et al., 2018). Moreover, the DMR assay appears to detect most GPCR-initiated signaling pathways related to activation of the four major G protein subtypes: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ (Grundmann et al., 2018). Thus, the approach has general applicability over a wide range of primary cells.

Despite the positive features of label-free DMR assays highlighted above, it is questionable if the approach will revolutionize the drug discovery process. Although false-positive hits are commonly found in any high-throughput screening campaign, counter screens conducted in parallel and other follow-up validation experiments usually pick up these false hits quite easily. Thus, failures of drug candidates in preclinical and clinical development are rarely related to false-positive hit compounds. In addition, not all biologic events result in robust redistribution within the cells and thus might not be detected in the DMR assay. Examples of GPCR events that are not detected in the DMR assay are receptor-recruitment of β-arrestins and β-arrestin–mediated signaling (Grundmann et al., 2018). Therefore, before determining whether the DMR assay can be used to detect new specific cellular functions, a decent amount of exploratory work and optimization is needed.

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Authorship Contributions

Wrote or contributed to the writing of the manuscript: Thomsen.

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