



High-throughput Meets Visualization: a Microscopy-based CRISPR Screening Platform in Ciliary Biology*

HE Ran**, XU Xiao-Han**, LI Jian-Chao***

(Innovation Centre of Ministry of Education for Development and Diseases, School of Medicine, South China University of Technology, Guangzhou 510006, China)

Abstract While pooled CRISPR screens have revolutionized functional genomics by enabling genome-scale interrogation, they have long faced a fundamental compatibility challenge with high-resolution microscopy-based phenotypes. The core issue lies in the technical disconnect between pooled screening formats, where cells are typically analyzed in bulk populations, and microscopy approaches that require spatial resolution and single-cell visualization. This limitation has created a significant blind spot in functional genomics, preventing researchers from systematically linking genetic perturbations to detailed subcellular architectural changes. To bridge this critical technological gap, a recent landmark study published in *Developmental Cell* has unveiled an integrated microscopy-based CRISPR screening platform that represents a paradigm shift in the field. The most innovative breakthrough of the platform involves extending photoactivation-based labeling techniques to fixed, immunostained cells, thereby overcoming a key limitation of prior approaches that were restricted exclusively to live-cell reporter systems. This methodological advancement allows researchers to capture transient cellular states and preserve delicate subcellular structures that would otherwise be lost in live-cell imaging scenarios. By seamlessly integrating this enhanced labeling approach with sophisticated automated imaging workflows and high-content analysis pipelines, the system enables unprecedented high-throughput, organelle-level phenotypic profiling at true single-cell resolution. The platform's capabilities were demonstrated through its application to a comprehensive genome-wide screen for regulators of cilium assembly, a complex cellular process involving intricate cytoskeletal dynamics and membrane remodeling events. The screening results validated the platform's robustness by successfully recapitulating known ciliary machinery components while simultaneously uncovering novel regulatory factors. Most notably, the study identified SMIM27/TZMP1, a conserved transition zone micropore protein, as a previously unrecognized regulator of ciliary function. This discovery provides crucial insights into the molecular mechanisms governing ciliary transition zone barrier formation and selectivity. This accessible and versatile framework effectively overcomes longstanding bottlenecks in microscopy-based screening technologies, offering a generalizable strategy for dissecting organelle biology and cellular architecture at scale. The platform's ability to combine genome-wide screening power with detailed morphological analysis opens new avenues for investigating cellular responses to genetic perturbations across diverse biological contexts.

Key words optical screening, CRISPR screen, photoactivation, cilia, ciliopathy, SMIM27/TZMP1

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Pooled CRISPR screening is a cornerstone of functional genomics, yet traditional approaches relying on growth or flow cytometry lack compatibility with microscopy-based subcellular phenotyping^[1-4]. To address this, the Breslow lab at Yale University developed a microscopy-based platform compatible with commercially available microscopes

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** These authors contributed equally to this work.

*** Corresponding author.

Tel: 86-20-39381050, E-mail: lijch@scut.edu.cn

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and standard software, as recently reported in *Developmental Cell*^[5]. By leveraging photoactivation-based strategies, the system supports both live-cell imaging and fixed-cell immunofluorescence, enabling organelle-level genetic screening. Applied to ciliary biology, this strategy not only recapitulated known regulators but also uncovered a conserved transition zone microprotein SMIM27/TZMP1. This work bridges high-throughput genetics and high-resolution imaging to redefine functional analysis at subcellular scales. Herein, we synthesize the technical innovations and biological insights from Breslow lab's study, elucidating how their photoactivation strategy couples genotype to subcellular phenotypes, hoping to serve as a valuable reference for the researchers.

1 Photoactivation technology links phenotypes to genotypes

Primary cilia are microtubule-based protrusions originating from the centriole, which are broadly involved in signal transduction and developmental regulation. Systematic identification of ciliary proteins is critical for understanding ciliogenesis, protein trafficking, and ciliary disassembly, among others^[6-8]. To this end, the research team integrated

imaging microscopy with pooled CRISPR libraries to establish a high-throughput screening pipeline for ciliary factors. The core workflow is as follows. (1) Transducing human retinal pigment epithelial (hRPE1) cells with lentiviral single guide RNA (sgRNA) libraries, followed by fluorescently tagged ciliary markers for living cells or immunofluorescence staining of fixed specimens. (2) Cells were first analyzed using artificial intelligence (AI)-based automated image analysis to classify ciliary phenotypes (*e. g.*, cilium presence or absence). Subsequently, cells exhibiting the phenotype of interest were selectively photoactivated using a less than 4 s high-power 405 nm light emitting diode (LED) coupled with a digital micromirror device (DMD), which enabling the processing of approximately one million cells per day. (3) Phenotype-specific cell populations were enriched *via* fluorescence-activated cell sorting (FACS), coupled with sgRNA sequencing to identify candidate functional genes (Figure 1). This platform incorporates contemporary AI-based image analysis technology, thereby achieving high efficiency and full automation of the entire workflow.

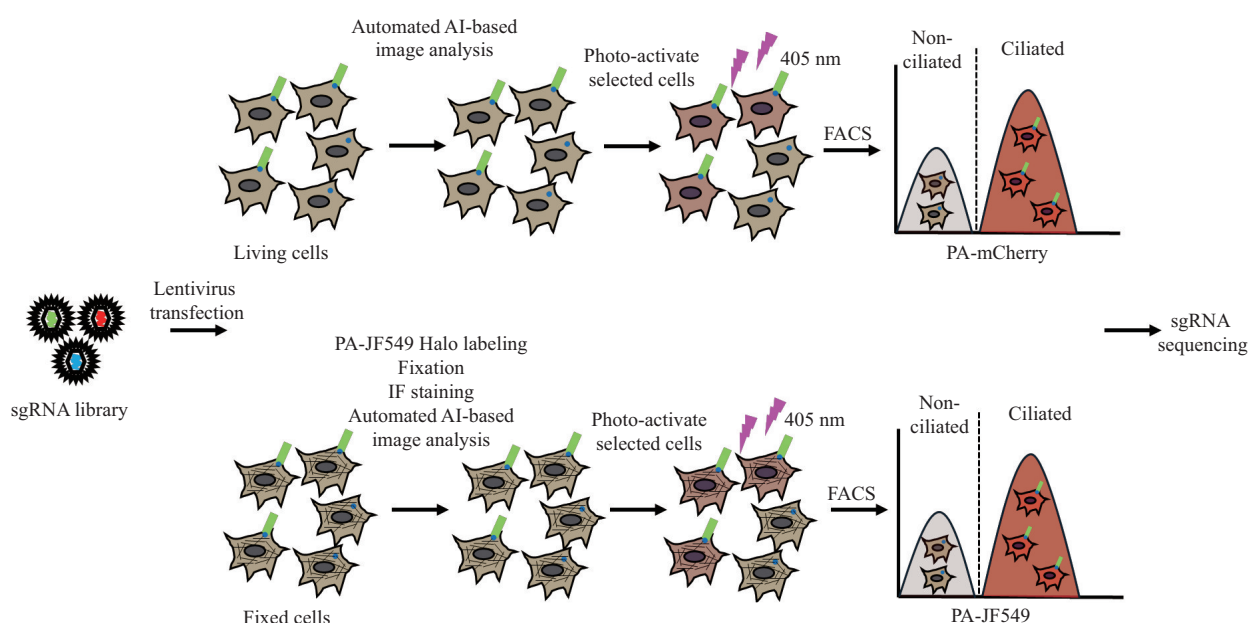


Fig. 1 The core workflow of the microscopy-based CRISPR screening platform

DMD: digital micromirror device; FACS: fluorescence-activated cell sorting.

Distinguishing it from prior strategies, the core innovation lies in two aspects. (1) Using engineered

cell lines expressing photoactivatable proteins (PA-mCherry) or utilizing small-molecule dyes (PA-

JF549)^[9], the system eliminates the dependence on live-cell fluorescent reporters by optimizing immunofluorescence protocols for fixed samples. These labeled cells are subsequently isolated *via* FACS, followed by sgRNA sequencing to quantify the enrichment of specific sgRNAs. (2) By relying exclusively on commercial microscope hardware and software rather than custom instrumentation, the platform demonstrates significant potential for broad dissemination.

2 From proof-of-concept to genome-wide scale: robust validation of the platform

To validate the performance of the platform, researchers conducted proof-of-concept experiments using the key ciliogenesis gene *RAB34* as a positive control^[10]. Results demonstrated that the system efficiently captured the cilium-loss phenotype induced by *RAB34* knockout and enabled rapid FACS enrichment of mutant cell populations *via* photoactivation labeling. Building on these pilot screening results, the team scaled up to a genome-wide screen using the Gattinara library (targeting 19 993 human genes with two sgRNA per gene)^[11], successfully enriching known essential ciliogenesis factors—members of the Intraflagellar Transport Complex B (IFT-B) family—with high concordance to prior Gene Ontology (GO) analyses and Hedgehog (Hh) pathway screens^[12-13]. To evaluate predictive performance, precision-recall and receiver operating characteristic (ROC) plots were constructed in MATLAB based on signed castLE scores (where positive and negative values correspond to predicted functional and non-functional effects, respectively). The results further confirmed the platform's robustness, demonstrating accurate identification of hit genes with low false positive rates and high sensitivity and specificity. Leveraging its low false-positive advantage, this platform delivers high-confidence candidate gene lists for fields requiring high-specificity target screening—particularly rare disease programs such as ciliopathies—thereby reducing the cost and uncertainty of subsequent functional validation.

3 Multi-dimensional profiling reveals novel regulators

Leveraging its compatibility with both live and

fixed cells, the platform enables dissection of gene function across ciliary phenotypes *via* diverse labeling strategies. Its key strengths are evident in three aspects. (1) Resolving cross-species and cell-type functional heterogeneity. For instance, *TMEM218* was identified as an essential gene for ciliogenesis in human hRPE1 cells but dispensable in mouse NIH-3T3 cells (Figure 2). (2) Characterizing functionally distinct classes of ciliary proteins. For example, depletion of the transition zone protein TMEM67 specifically disrupted ciliary membrane protein localization without blocking ciliogenesis^[14] (Figure 2). Using the ciliary GPCR (G protein-coupled receptor) HTR6 as a probe^[15], researchers identified the ER membrane protein complex (EMC) as specific regulators of GPCR trafficking that do not compromise ciliary structural integrity. They further found that knockout of m⁶A methyltransferase components METTL3 and VIRMA reduced HTR6 ciliary localization, implicating unappreciated roles of RNA modification in ciliary function (Figure 2). (3) Discovering novel regulators. The screen revealed that deletion of diphthamide biosynthesis factors DPH1/DPH5^[16] resulted in strongly increased ciliation even under serum-supplemented conditions and impaired serum-induced cilia disassembly, implicating diphthamide biosynthesis as a previously unrecognized contributor to cilium disassembly (Figure 2). Crucially, the platform identified SMIM27/TZMP1^[17], a conserved 55-amino acid single-pass transmembrane microprotein, which plays an essential role in transition zone assembly. Initially annotated as a non-coding locus near C9orf133/TOPORS, SMIM27/TZMP1 was only recently shown by ribosome profiling to encode a functional open reading frame (ORF). Due to the diminutive size, such microproteins eluded structural characterization and were long overlooked. Subsequent validation showed that SMIM27/TZMP1 interacts with the Meckel-Gruber syndrome (MKS) complex (including MKS1, B9D1/2, TCTN1/2/3, TMEM231, TMEM17 and CC2D2A), which is a multi-subunit ciliary gatekeeper localized at the ciliary transition zone. The MKS complex scaffolds the Y-shaped linker structures connecting the ciliary membrane to the underlying axoneme to establish the selective permeability barrier essential for Hedgehog signaling and ciliogenesis, with its dysfunction underlining the pathogenesis of Meckel-Gruber and related ciliopathies (Figure 2).

Moreover, SMIM27/TZMP1 was found to be necessary for ciliary motility in epidermal multiciliated cells (MCCs) and proper left-right patterning in *Xenopus* models. The platform's phenotype-first design, which is agnostic to prior genomic annotation, directly addresses a systemic blind spot in the field of transmembrane microprotein discovery and uniquely positions it to capture a wealth

of previously functionally invisible elements. Collectively, rather than merely accelerating gene discovery, this platform enables multidimensional phenotypic dissection, integrating cryptic microproteins and non-canonical modifications into ciliary regulatory networks, thereby reshaping mechanistic understanding and identifying new therapeutic targets for ciliopathies.

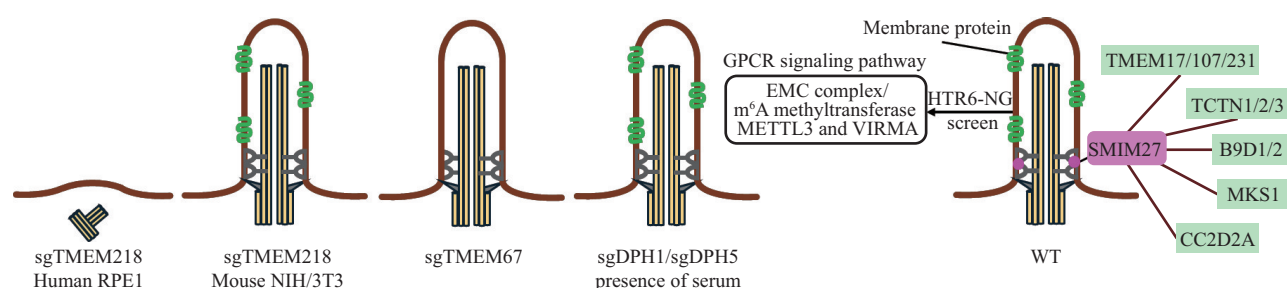


Fig. 2 Multi-dimensional dissection of ciliary gene function via integrated labeling

GPCR: G protein-coupled receptor; EMC: ER membrane protein complex.

4 Discussion

High-throughput CRISPR screening serves as a pivotal tool in functional genomics, and the Breslow team's integration of microscopy visualization now enables systematic profiling of subcellular structures (such as cilia), achieving a synergistic integration of throughput and resolution. Beyond uncovering novel ciliary machinery, this platform holds profound implications for understanding developmental diseases. From a clinical perspective, the identification of SMIM27/TZMP1 as a core component of the MKS complex establishes it as a compelling candidate gene for syndromic ciliopathies, offering new insights into diagnosis for human developmental disorders. Methodologically, while validated in ciliary biology, this commercially accessible framework can be readily generalized to dissect the functional genomics of other diverse subcellular organelles. Nevertheless, limitations persist: the use of only two sgRNAs per gene limits coverage and may increase false-negative rates; and applications in 3D cellular models or tissues are still challenging due to the limited precision in the *z* axis of DMD-based photoactivation.

Overall, this platform provides an omics-scale readout of subcellular morphology and

supramolecular assembly states, resolving the high-dimensional phenotypic landscape of the cell. Its hardware configuration and experimental workflow are amenable to adoption by a broad range of laboratories for analogous screens targeting diverse organelles, thereby moving beyond reliance on highly customized systems. With appropriate adaptation, the framework is theoretically transferable to other stably imageable subcellular structures or organelles, provided that robust imaging parameters can be defined (*e. g.*, area, length, fragmentation, colocalization, polarity distribution), thus facilitating the establishment of reusable standardized pipelines. Current limitations restrict its applicability to 3D systems such as thick tissues, deep regions of organoids, and whole embryos. Furthermore, target sets with high sensitivity to false negatives may necessitate greater library depth or additional sgRNA/allelic configurations to enhance detection power. Despite these constraints, the platform offers a versatile new strategy for investigating organelle structure and function, deepening insights into organelle biology.

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高通量与可视化的结合：基于显微镜的 纤毛基因筛选平台开发*

何 然** 徐晓涵** 李健潮***

(华南理工大学医学院, 教育部发育与疾病创新中心, 广州 510006)

摘要 虽然混合 CRISPR 筛选技术已能实现全基因组尺度的基因功能图谱绘制, 但长期以来仍难以与基于显微镜成像的高分辨率表型有效兼容。这一瓶颈严重阻碍了人们在单细胞层面系统解析基因扰动对细胞器结构与功能影响的能力。为了跨越这一技术鸿沟, 近期发表于 *Developmental Cell* 的一项研究系统构建并阐述了一种基于显微镜成像的 CRISPR 筛选平台。该平台的核心突破在于将光激活标记技术扩展应用于固定的免疫染色细胞, 克服了以往方法依赖于活细胞报告基因而无法捕捉瞬时或精细亚细胞结构的关键局限。同时, 研究人员整合了高度自动化的细胞成像 workflow, 实现了单细胞分辨率下对细胞器形态及空间分布特征的高通量表型谱分析。通过将该平台应用于纤毛组装调节因子的全基因组筛选, 研究人员不仅重现了已知的纤毛组装和调控机制, 还意外发现了包括保守的过渡区微蛋白 SMIM27/TZMP1 在内的新型调控因子, 揭示了其在纤毛过渡区屏障功能中的关键作用。这一技术框架操作相对简便且具备较高的普适性, 有效克服了显微成像筛选长期面临的通量与分辨率难以兼得的桎梏, 为未来大规模系统性剖析细胞器生物学功能、细胞架构维持机制以及相关疾病的致病基础提供了强有力的通用策略。

关键词 光学筛选, CRISPR 筛选, 光激活, 纤毛, 纤毛病, SMIM27/TZMP1

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** 并列第一作者。

*** 通讯联系人。

Tel: 020-39381050, E-mail: lijch@scut.edu.cn

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