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ARTICLE

Lipid Mapping of Cell Mitosis by Non-covalent Migratory Fluorescence Labelling

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Abstract The concept of non-covalent migratory fluorescence labelling is introduced to spatially and temporally map intracellular lipids throughout a cell division cycle. This hands-off approach utilizes a small molecule BF₂-azadipyromethene fluorophore, NM-ER, to first label the nuclear membrane and endoplasmic reticulum of cells at interphase but which can migrate with the lipid components of these structures throughout mitosis as they disassemble, redistribute and reassemble prior to daughter cell separation. Through this unique approach to image capture, key prometaphase events such as lipid intrusion into the nucleus and nuclear membrane disassembly are observable, as are the stages of nuclear membrane reassembly in telophase and lipid distribution during cytokinesis. When used alone NM-ER can distinguish each phase of cell mitosis from lipid staining patterns, is compatible with STED super resolution imaging, and with an emission max of 648 nm makes it useable with other common GFP and nuclear DNA stains. The non-covalent NM-ER label remains associated with the originating lipid components as they undergo architectural reorganizations and changes in subcellular localization associated with mitosis. As lipid-based cell structures are influenced by numerous biological processes and mechanical forces, our approach to fluorescence imaging could offer novel perspectives into their different roles.

INTRODUCTION

Lipids, alongside amino acids, nucleotides and carbohydrates are the underpinning building blocks of cells, though lipids differ in how they are employed in the construction of their higher order structures. The macromolecular protein, DNA strand or polysaccharides being formed from cell programmed covalent bond formation, whereas in the case of lipids it is a collective of weak Van de Waal, hydrophobic forces and phase separations that drive the assembly of their higher ordered structures (without the formation of covalent bonds).¹ Another point of difference is that while lipids can be broadly grouped as phospholipids, glycerides, sphingolipids or sterols they have structural diversity greater than that of the other biomolecular classes.² As cells produce tens of thousands of different lipid

species performing key roles within membrane and organelle structures, signaling, and energy storage it makes their study particularly challenging.³ The dramatic breakdown and reforming of lipid comprised membrane structures such as the endoplasmic reticulum (ER) and nuclear membrane (NM) that occurs during cell division can make this challenge even more pronounced.⁴ Fluorescence microscopy and lipidomic mass spectrometry are two approaches often employed which can give complementary information with the former suitable for visualizing structures and the latter enabling the identification of specific lipids.⁵

Lipidomics has provided the ability to identify individual lipid species, but visualizing lipid dynamics remains a persistent challenge which could be addressed by fluorescence microscopy.⁶ Most often, fluorescence labelling for microscopy is achieved through the direct covalent linkage (or strong specific binding) of a targeted molecular fluorophore or genetically expressed fluorescent protein to a cellular feature of interest.⁷ However, as lipids are relatively small molecules when compared to proteins, covalently linking them to a fluorescent probe could overly alter their molecular structure thereby perturbing their natural functions and localizations.⁸ What

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The supporting information is available free of charge at <https://pubs.acs.org/.....>. Additional representative microscopy images shown in Figures S1 – S8 and associated Movies S1– S13 of Figure images.



remains relatively unexplored is the potential to utilize a non-covalent fluorescence labelling of lipid based cellular structures to track their changes over time. Uniquely, this would require that the non-covalent labels remain associated with the components of the originating lipid structure as they undergo architectural reorganizations and changes of their subcellular localizations. As lipid structures and localization are influenced by both biological processes and mechanical forces, such an imaging tool could offer a novel perspective on lipid behavior in live cells.

Despite their central importance, an understanding of lipid participation in pivotal biological events such as cell division remains underdeveloped.⁹ Lipidomic mass spectrometry has provided key insights into the spatial and temporal identification of specific lipids regulated during cell division. Indeed, elegant studies by Eggert *et al.*, revealed that lipid composition changes at different mitotic phases, suggesting that for reasons not yet fully understood, specific lipids play distinct roles at different stages.¹⁰ This is in strong contrast with the extensive knowledge of DNA and microtubular changes which have been extensively studied (Figure 1). In a similar manner, cell replication causes dramatic changes to lipids structures and spatial distribution during each phase of mitosis. At the outset of mitosis (interphase) the NM and ER are intact, yet they will entirely deconstruct during pro(meta)phase, following which there are continuous lipid reorganizational changes through anaphase and telophase, with the NM and ER both being fully reconstructed in time for the completion of cytokinesis. Previously reported approaches for the microscopy imaging of lipids during mitosis have employed fluorescent ER membrane bound proteins such as STIM1 and Sec61b.¹¹ In this work we have developed the novel use of a single intracellular lipid favoring molecular fluorophore to map the dramatic changes in lipidic structures throughout cell division.

In a recent study, we introduced the BF₂-azadipyromethene fluorophore NM-ER, which non-specifically stains intracellular lipid membranes such as the NM and ER of interphase cells through non-covalent association with the lipids within these connected structures (Figure 1, (interphase cell image, prior work), Figure 2 (structure)).¹² For the next stage of this work, the use of this fluorophore to follow the dramatic structural changes that occur to both the NM and ER during open mitosis in higher eukaryotes has been investigated.¹³ As the NM and ER disassemble upon mitotic entry and reform upon completion to reestablish nucleocytoplasmic compartmentalization it would be of significant value if these events could be tracked from start to finish using a single molecular probe. As such, if NM-ER remained associated with the dispersed lipids throughout the deconstruction and reformation of these structures, this would enable spatiotemporal mapping of their lipid distributions throughout cell mitosis (Figure 1, this work). Once created, a mitotic lipid distribution map could complement existing DNA and microtubule markers, offering a less disruptive alternative that avoids potential fluorophore-induced toxicity associated with DNA and microtubule binding. Moreover, NM-ER is compatible with the super-resolution microscopy technique STED (stimulated emission depletion microscopy) so it could

offer deeper insights into the roles of lipids during the complex sequence of events during cell division.¹⁴ DOI: 10.1039/D5TB01947H

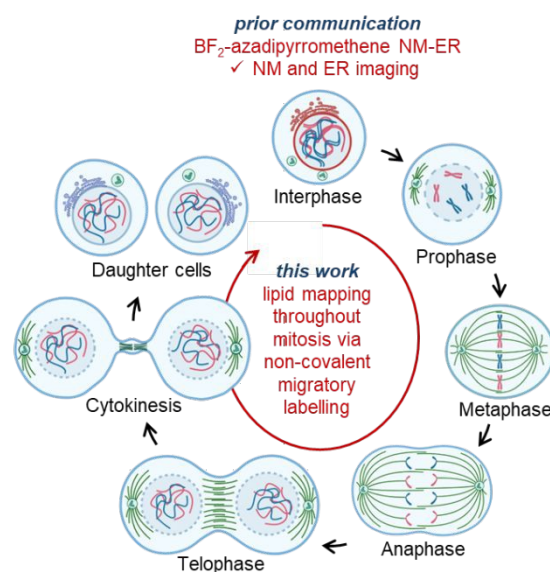


Figure 1. Mapping of cell division phases through the recognizable changes in DNA and microtubules and in this work potentially through their associated lipid distribution patterns. This schematic represents the DNA and microtubular organization at different phases of cell mitosis from a cell in interphase to its division onto two daughter cells. In this work, fluorophore NM-ER which has been shown to non-covalently label lipids in the nuclear membrane (NM) and the endoplasmic reticulum (ER) of cells at interphase (top cell) will be explored as a means of microscopy imaging these lipids throughout mitosis by migratory fluorescence labelling.

RESULTS AND DISCUSSION

Characterization of BF₂-azadipyromethene fluorophore NM-ER for lipid imaging

The BF₂-azadipyromethene fluorophore class has become increasingly popular in fundamental and applied imaging applications.¹⁵ Advantageously, their emissions can be refined to meet specific needs, are highly photostable and have shown no toxicity issues when used *in vitro* or *in vivo*.¹⁶ To achieve the imaging objective for NM-ER, it was designed to follow intracellular lipid structures by migrating alongside them as they undergo cellular reorganization (Figure 2A, schematic illustration). Structurally NM-ER is overall charge neutral, has the simplest of BF₂ chelates and has a pyrrole phenyl substituents for optimal emission wavelengths (Figure 2). It has only moderate solubility in phosphate buffered saline (PBS), which is achieved through the inclusion of the two primary alcohol substituents at the β -pyrrole positions (Figure 2B). As 1-alkanols are recognized to be highly effective at assembling inside lipid bilayers, with the hydroxyl group hydrogen bonded to the carbonyls of lipid headgroups, it was thought plausible that the bis-1-alkanols of NM-ER would favor accumulation within intracellular membranes such as ER and NM.¹⁷ To



illustrate the potential for this association, a comparison of the emission properties of NM-ER in PBS alone and in solutions containing the representative triacylglyceride glyceryl trioleate, or a mixed lipid system of L- α -phosphatidylcholine and cholesterol were investigated.^{3c,18} As expected a weak emission centered at 647 nm was recorded in PBS alone, and while both lipid-containing solutions showed a negligible change in wavelength emission maxima (648/647 nm) they had a 69- and 63-fold increase in intensity respectively (Figure 2B). These initial results illustrated that when NM-ER is associated with aqueous lipid microenvironments its emission is significantly enhanced, but the unknown question for this work being if this association could be utilized to image lipid structures as they disassemble and reassemble during cell mitosis (Figure 2A). If this proved to be the case, then the spatiotemporal image capture of lipid associated structures at each phase of mitosis could be imageable using a single molecular fluorophore.

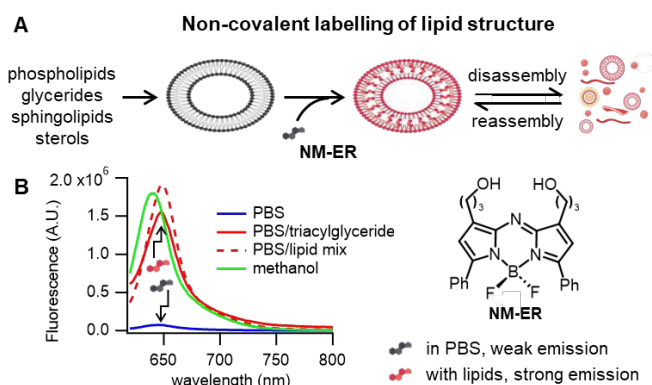


Figure 2. Lipid responses of NM-ER. (A) Concept schematic of dynamic lipid structure imaging through non-covalent migratory fluorescence labelling. (B) Emission spectra of NM-ER at 5 μ M in phosphate buffered saline (PBS, blue trace, λ_{max} 647 nm), in PBS/glyceryl trioleate (solid red trace, λ_{max} 648 nm), in PBS/L- α -phosphatidylcholine and cholesterol mixture (dotted red trace, λ_{max} 647 nm) and in MeOH (green trace, λ_{max} 638 nm) upon excitation at 590 nm. NM-ER structure and lipid fluorescence response.

Prolonged live cell imaging with NM-ER

To first determine if intracellular NM-ER emission could be sustained throughout mitosis, live cell experiments were conducted in which the progression from parent to daughter cells was imaged. Encouragingly when 0.5 μ M of fluorophore was added to HeLa Kyoto (HeLa) cells, it was found that emission was readily detected throughout each phase of the replication process with the daughter cells each having comparable fluorescence intensity as the parent (Figure 3, SI Movie S1, Supporting Figure S1 and Movie S2 for additional experiment with DNA co-stained). These prolonged 4 h timelapses in live cells indicated that NM-ER could be used to follow lipid-induced emission without impeding mitosis.

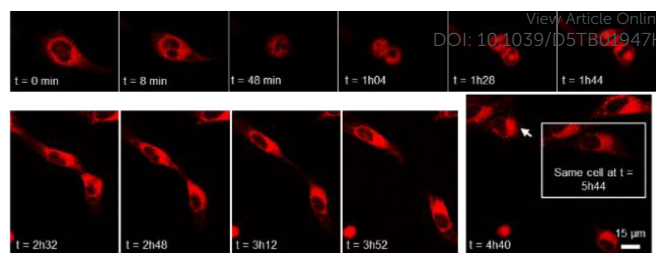


Figure 3. Time lapse of NM-ER (0.5 μ M concentration) fluorescence imaging of an individual HeLa Kyoto cell undergoing mitosis to two daughter cells over 4 h. Confocal microscope images acquired with a 20x objective (SI Movie S1).

Interphase lipid membrane imaging with NM-ER

Next, for a more detailed analysis of lipid distribution throughout mitosis using NM-ER, the representative cancerous and macrophage cell lines HeLa, MDA-MB 231 (MDA) and RAW 264.7 (RAW) were employed. As previously reported for cells at interphase, NM-ER stains several key lipidic structures including the NM and the ER.¹² Notably at interphase in all three cell lines the NM was seen as a distinguishable line surrounding the nucleus and ER staining pattern was of tubule-like structures with mitochondria also observed (SI Figure S2). Prior literature, using cells expressing the fluorescent ER bound proteins YFP-STIM1 or GFP-Sec61 β have described ER lipid structures as either sheet- or tubule-like at interphase and that an interconvertible continuum may exist between both forms.^{19,20}

Mapping lipids during mitosis

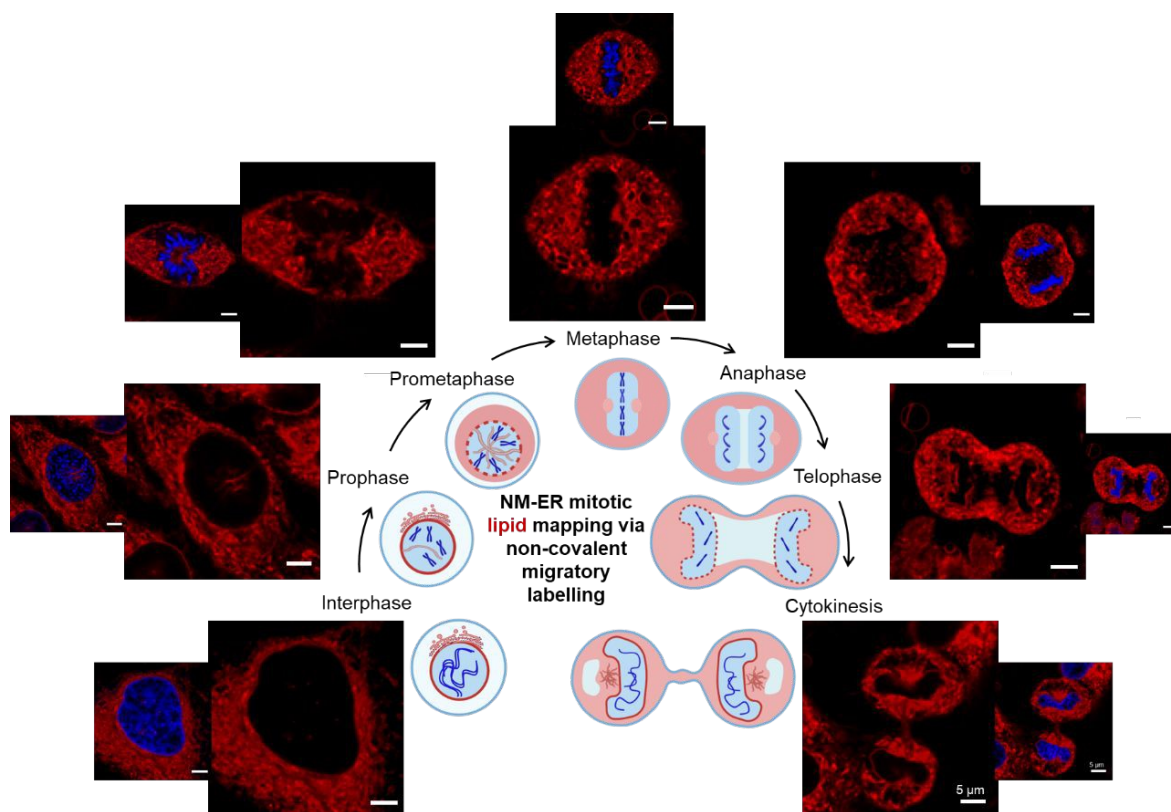
Initially, cells undergoing division were incubated with either NM-ER alone or co-stained with the DNA marker Hoechst. With images generated from NM-ER alone, each of the mitotic phases was clearly distinguishable from one another through their characteristic patterns of fluorescent lipid areas and lipid free “negative spaces” (Figure 4 and SI Movie S3 for 3D videos of each phase). Known features of mitosis could be gleaned from these images such as during prophase, while the NM remains intact, the first ingress of a lipid tentacle into the nucleus can be observed originating from the NM. As prophase progresses into prometaphase and the NM breaks down, this lipid intrusion expands, forming a squid-like series of branched tubules interposed between the chromosomes (Figure 4, prometaphase). In metaphase, as the cells round up, lipids occupy the entire intracellular space except for the metaphase plate, where the chromosomes are lined up, giving a dumbbell-shaped outline (Figure 4, metaphase). As sister chromatids are pulled apart in anaphase, the interspace zone which is essentially free of lipids, enlarges in size surrounded by lipids resulting in the lipid free negative space taking on a butterfly wings shaped pattern (Figure 4, anaphase). As the NM is reformed during telophase, this interspace gradually fills with lipids as the cleavage furrow deepens. A lipid-free region is consistently observed at the poles of the two future daughter cells (opposite to the junction area) with a high concentration of lipids around the reforming nuclei. During cytokinesis, the



lipid-rich intracellular bridge connects the near-separated daughter cells that resemble a pair of eyes as the chromatin discs at the center of each cell are surrounded by lipids (Figure 4, cytokinesis). The DNA co-stain proved helpful to confirm the identity of each mitotic phase (prophase, prometaphase, metaphase, anaphase, telophase) and cytokinesis of individual

cells, however with experience the NM-ER pattern alone became sufficient to make this determination (Figure 4, SI Movie S3). With the phases clearly identified and distinguishable from each other, we next examined each in more detail to investigate dynamic changes to their lipid structural features.

Figure 4. Lipid mapping with NM-ER throughout cell mitosis by non-covalent migratory fluorescence labeling. Larger cell images show NM-ER (red) fluorescence patterns at each phase of cell mitosis. The smaller images correspond to the same cell with Hoechst co-registered DNA (blue).



Cartoons illustrate the identifiable pattern shapes for lipids (red regions) for each phase with DNA as dark blue colored regions) and negative space areas with no fluorescence shown in grey. Scale bar 5 μm . 3D stacks of each mitotic phase can be visualized in SI Movie S3.

Disassembly of the NM and ER during prometaphase

Prometaphase is a transitional phase of mitosis characterized by the disassembly of the NM and ER, allowing spindle microtubules to access the condensed chromosomes.^{4a,21} During this stage, kinetochore complexes form at the centromeric regions of each chromosome, serving as anchoring sites for spindle fibers originating from opposite centrosomes. Once attached, microtubules facilitate the movement of chromosomes toward the metaphase plate. Viewing the prometaphase from a lipid standpoint with confocal microscopy, the onset of mitosis can be distinguished by a tentacle-like intrusion of lipids, emanating from the NM, into the nucleus body. When co-stained with Hoechst, these lipid tentacles closely follow the contours of the condensed chromosomes prior to their migration to the metaphase plate (Figure 5A, SI Movie S4, for other examples see SI Figure S3). These intricate structures together with the remaining sections

of the NM, were better visualized with super-resolution STED imaging. This enhanced the resolution by a factor of three, providing a clearer view of both the origins of the lipids and the tubular network itself (Figure 5A). The STED measurements of individual intruding tubules were comparable with the NM of an adjacent interphase cell (105 nm). While not apparent from the NM-ER images, it could be understood that the lipids alone could not exert the mechanical forces necessary to drive chromosome condensation and separation and that other non-lipid structures must be closely associated with these lipids (see later).

It could also be observed that as NM disassembly proceeded, in tandem the intranuclear lipid intrusion grew. The visual hallmark of this disassembly is that the NM surface loses its smooth continuous appearance in interphase, developing irregular contours, and becoming disjointed. Figure 5B shows an example 3D image of a section of continuous NM in a cell at the interphase stage, with a neighboring prometaphase cell in the



same field of view being fenestrated and disjointed. By completion of prometaphase all recognizable structural features of the NM and ER have disappeared, with their lipids dispersed as metaphase emerges (SI Figure S3).

centrosomes have a concentration of densely packed lipids (Figure S4). This gives the distinctive contrasting non-fluorescent regions (negative shapes) through which both metaphase and anaphase phases can be readily identified (Figure 4).

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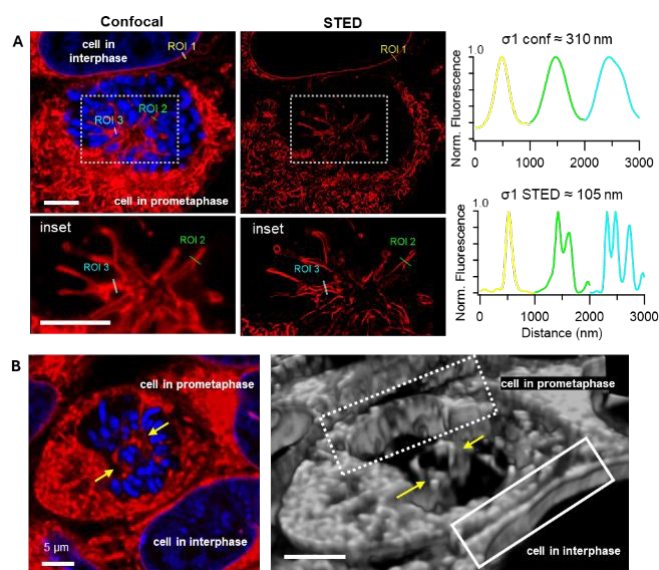


Figure 5. Observing nuclear lipid intrusion and nuclear membrane disassembly during prometaphase in HeLa cells. (A) Confocal and STED images of two cells, one in interphase and one in prometaphase, imaged with NM-ER (red) and DNA co-registered with Hoechst (blue). Early-stage lipid intrusions into the nucleus of the cell in prometaphase highlighted within boxes and shown as expansions below the full images. Comparative confocal and STED fluorescence intensity profiles of three different ROIs as indicated in the images (ROI 1 nuclear membrane of interphase cell, ROI 2 and 3 nuclear lipid channels of prometaphase cell), see SI Movie S4 for 3D view and for images from additional experiments see SI Figure S3. (B) Single frame of a 3D reconstruction image of two cells, one in interphase and one in prometaphase, imaged with NM-ER (red) and DNA co-registered with Hoechst (blue) showing a section of disassembling nuclear membrane and lipid nuclear intrusion indicated by the yellow arrows (left image). For clarity the NM-ER channel of the same two cells is shown in black and white with the disassembling nuclear membrane of the prometaphase cell indicated within the dashed box and as comparison an intact nuclear membrane of the adjacent interphase cell within the solid box. Scale bars 5µm.

Key cellular tasks during metaphase and anaphase are the movement of chromosomes to the metaphase plate and their attachment to spindle microtubules via kinetochores. These microtubules originate from opposite centrosomes, ensuring bipolar attachment, which is essential for the equal distribution of genetic material. During anaphase, motor protein shortening of the microtubules forces separation of the sister chromatids and they are pulled toward the opposite poles of the cell. Concurrently, the non-kinetochore microtubules elongate, pushing the cell poles further apart and contributing to cell elongation. Remarkably, the central region of the cell in which much of this activity occurs remains essentially devoid of lipids whereas the outer circumference and regions surrounding the

Reassembly of the NM during telophase

Telophase, the reverse of pro(meta)phase, is the known cycle time point when the NMs begin to form around the separated chromatin discs which will become the two future daughter cell nuclei. Importantly with respect to the reassembly of the NM, previous reports have shown that there are three spatial edge regions of the chromatin discs which are termed the inner and outer cores and non-core regions (Figure 6A, schematic).²² It has been reported in the literature that NM formation may occur in either a synchronous or asynchronous manner, during which different sections of the membrane form in a specific temporal order. For example in HeLa cells, an asynchronous assembly has been noted in which the membrane forms first at the non-core positions, then at the outer core and finally at the inner-core.²² Using NM-ER emission as a guide, HeLa cells in telophase were examined to detect evidence of this temporal regulation of NM formation. Through confocal and STED microscopy, cells were identifiable at each of the three steps of NE formation confirming an asynchronous assembly around the chromatin discs for this cell line (Figure 6 and SI Movie S6 for the associated 3D movies, for additional images see Figure S4). Timelapse microscopy of a single live cell over 20 min was employed to confirm that following membrane assembly in the non-core region, the outer-core was next to be addressed (Figure 6D-E, SI Movie S7). The late inner core formation has been attributed to microtubule detachment from the chromosomes prior to this section of the membrane being completed.²³ Remarkably, nuclear pore (NP) complexes were observable in the first assembled non-core membranes prior to completion of the core membrane regions through the use of the NP stain WGA-CF594 in fixed cells (Figure S5). It is known that NP complexes can assemble concurrently with the membrane at the non-core chromatin disc positions indicating that some lipid sub-structures used may contain preassembled NP complexes.²⁴ This study of lipids during telophase underscores the potential for using NM-ER to observe the temporal regulation of membrane recruitment to the chromatin surface.



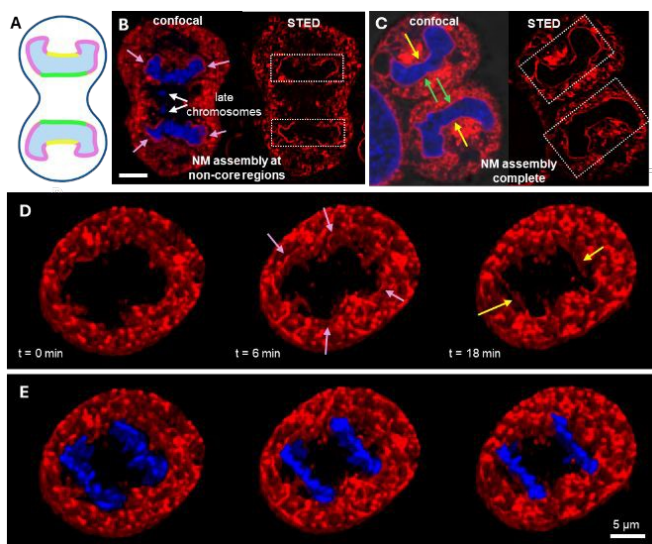


Figure 6. Identification of the three periods of nuclear membrane reassembly around the chromatin discs in HeLa cells using NM-ER (red) with DNA co-stained with Hoechst (blue). (A) Cartoon showing non-core (purple), outer-core (yellow) and inner-core (green) chromatin disc regions. (B) Confocal and STED images of a cell showing partially reassembled NM (purple arrows in confocal image and dashed boxes in STED image) at the non-core regions of the chromatin discs. Late chromosomes indicated by white arrows. (C) Confocal and STED images of a cell showing completely reassembled NM (yellow and green arrows in confocal image and dashed boxes in STED image) at both non-core and core regions of the chromatin discs. (D) Timelapse of confocal images over 15 min showing first the non-core assembly (purple arrows), followed by the outer-core assembly (yellow arrows) of the NM. (E) The same image sequence as in (D) with DNA co-stain included. Scale bars 5 μm . For images from additional experiments, see SI Figure S4 and movies S6–8).

Lipid accumulation at the cleavage furrow during cytokinesis

Cytokinesis, as the final step of the cycle during which the cytoplasm divides to form two daughter cells, begins with the formation of a cleavage furrow during telophase that deepens until the parent cell is pinched into two separate cells.²⁵ Lipids are known to play multifaceted roles in cytokinesis, particularly in membrane remodeling, signaling, and the mechanical stability of dividing cells.²⁶ As was the case for each phase of mitosis, NM-ER emission persisted into cytokinesis with the lipid contents of the cytosol, the fully reassembled NMs, and the cleavage furrow clearly visible (Figure 7A, for 3D z-stack see SI Movie S9).

The midbody is a microtubule-rich structure which forms in the intercellular bridge between the two daughter cells during late cytokinesis and plays a pivotal role in orchestrating the final abscission event.²⁷ Examination of NM-ER images acquired nearing the final moments prior to abscission showed the accumulation of lipids on either side of the midbody which itself could be recognized as a dark zone situated between these two concentrated lipid areas (Figure 7B, for 3D z-stack see SI Movie S9).

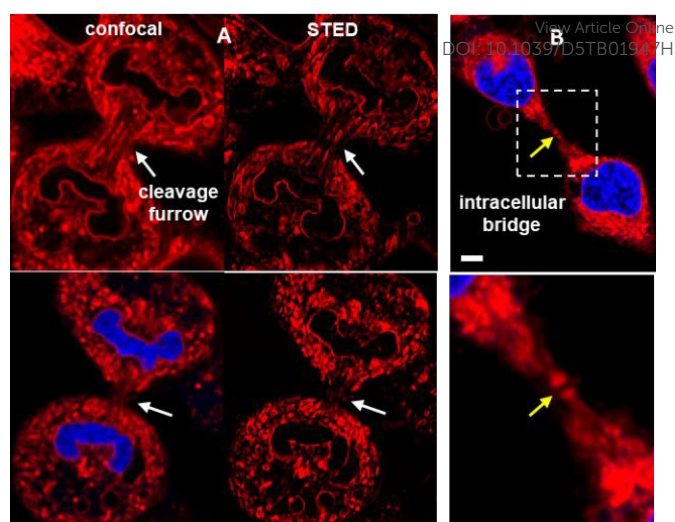


Figure 7. Confocal and STED image capture of lipids during cytokinesis in HeLa cells using NM-ER (red) with DNA co-stained with Hoechst (blue). (A) Representative confocal and STED images of a cell at early cytokinesis with a completed NM and lipid accumulation at the cleavage furrow (white arrows). (B) Confocal images of a cell at late cytokinesis with distinctive lipid accumulation at the intracellular bridge (boxed area) and with midbody indicated (yellow arrows) with lipids flanking the midbody shown in expanded view of the boxed area. Scale bars 5 μm . SI Movie S9 and SI Figure S6 for additional examples.

Co-registering NM-ER with microtubules during mitosis

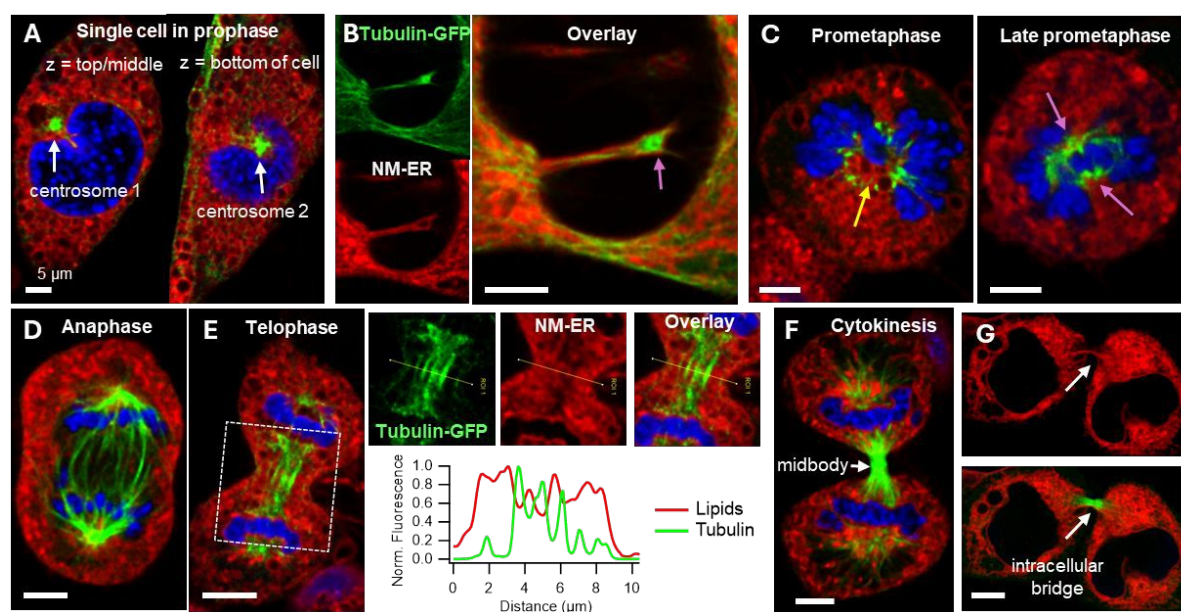
Microtubules play a crucial role in ensuring accurate chromosome segregation during cell mitosis. These dynamic, cylindrical structures, composed of α - and β -tubulin dimers, assemble into the mitotic spindle which is responsible for aligning and separating the chromosomes.²⁸ At each phase of cell replication, they exert distinct pushing and pulling mechanical forces generated by their ability to rapidly grow (polymerize) and shrink (depolymerize). Following mitotic spindle disassembly, a subset of microtubules reorganize into the central spindle and midzone to control the cleavage furrow and guide membrane trafficking events essential for the final physical separation of daughter cells during cytokinesis.²⁹ As such, the association between lipids and microtubules is of fundamental interest as lipids balance intracellular forces and microtubules exert dynamic forces critical to cell division. In order to co-register NM-ER stained lipids with microtubules and centrosome boundaries, CellLight tubulin-GFP (a fusion construct of human tubulin and emGFP) was used to stain tubulin in a live cell compatible manner.³⁰ Notably, while lipids and centrosomes were closely associated throughout mitosis, microtubules varied according to the mitotic phase. Upon examination of cells co-stained with NM-ER and tubulin-GFP, it became apparent that centrosomes resided within a pocket of lipids which was most apparent in prophase and metaphase (Figure 8C).



The first lipid intrusions into the nucleus detected by NM-ER in prometaphase occur in regions near the centrosomes, where the accompanying microtubules are also enclosed by lipids (Figure 8A and B, SI Movie S11). As the nuclear intrusion of lipids grows throughout prometaphase this was closely associated with the expansion of microtubules into the nucleus where attachment to the chromosomes occurs (Figure 8C, SI Movie S11). During metaphase and anaphase microtubules transect into the interspace between the two centrosomes, however, this central region is essentially devoid of lipids while

the centrosomes themselves remain surrounded by lipids (Figure 8D). As the cleavage furrow emerges and deepens during telophase, lipids infiltrate the interspace and have close spatial proximity to the microtubules as confirmed by line intensity emission profiles for both fluorophores (Figure 8E). As expected, cells in cytokinesis had a high concentration of microtubules through the intercellular bridge between the daughter cells with NM-ER showing the presence of surrounding lipids (Figure 8F, G, SI Movie S11).

Figure 8. Co-registration of lipids with microtubules (tubulin-GFP) at different cycle phases of mitosis and cytokinesis. HeLa cells expressing tubulin-GFP (green) with Hoechst labelled DNA (blue) and NM-ER labelled lipids (red). (A) Two images of a single cell in prophase at different z positions showing the two centrosomes connected by microtubules and lipids. (B) Expanded view of microtubules surrounded by lipids with one cell



centrosome in view. (C) Images of two different cells in prometaphase; on the left, the intrusion of lipids into the nucleus is closely associated with the centrosomes, the spindle forming regions and between chromosomes. The right cell image shows the forming spindles and associated centrosomes are surrounded by lipids. (D) Cell in anaphase with lipids mostly confined to the cell peripheral regions with the interspace being relatively devoid of lipids. (E) Cell in telophase showing lipids in close association with microtubules within the cleavage furrow, insets showing expanded views of tubulin-GFP and NM-ER images of boxed cell area, and fluorescence intensity profile plots of ROI line showing overlapping association of microtubules and lipids. (F) Cell in cytokinesis showing lipid, DNA and microtubule distribution in the daughter cells and the midbody closely abutted to the plasma membrane of the intercellular bridge between the cells. (G) Illustration using NM-ER only (top), and together with tubulin-GFP (bottom), of the presence of lipids within the intracellular bridge during cytokinesis. Scale bars 5 μ m

Co-registering NM-ER with mitochondria during mitosis

Mitochondria change shape and outer lipid membrane components during mitosis, so it was considered likely that NM-ER could also stain their outer membranes. Published studies have shown that mitochondrial distribution is regulated by the microtubule cytoskeleton during division and that their distribution changes with each phase.³¹ To investigate the lipid/mitochondria relationship, HeLa cells at each phase of mitosis were co-stained with NM-ER and Mito-Tracker green and the distribution of both was examined (Figure S7). Specifically, mitochondria were not observed in many active lipid areas such as the nuclear lipid intrusions of prometaphase or surrounding centrosomes during metaphase/anaphase nor in the reforming NM during telophase. In metaphase the

mitochondria were observed in the cytoplasm around the metaphase plate though excluded from the central region of the cell, while in telophase they were in the cleavage furrow and during cytokinesis in the intracellular bridge, which is consistent with previous reports.³²

NM-ER advantage over DNA staining in live cell imaging

In cultured HeLa cells, aberrant tripolar mitotic cells were occasionally observed during live cell experiments.³³ As DNA binders such as Hoechst are known promoters of tripolar mitosis, we took advantage of NM-ER to examine lipid distributions during this abnormal cell division.³⁴ In such multipolar divisions the lipid distributions through each phase, as marked by NM-ER, were like that of a normal bipolar division



albeit in triplicate. In prophase three lipid-surrounded centrosomes were observed which progressed through chromosome separation in metaphase and NE reassembly occurring in telophase (Figure 9A-C, SI Movies S12). Finally, upon entering cytokinesis abscission resulted in the production of three daughter cells (Figure 9D).

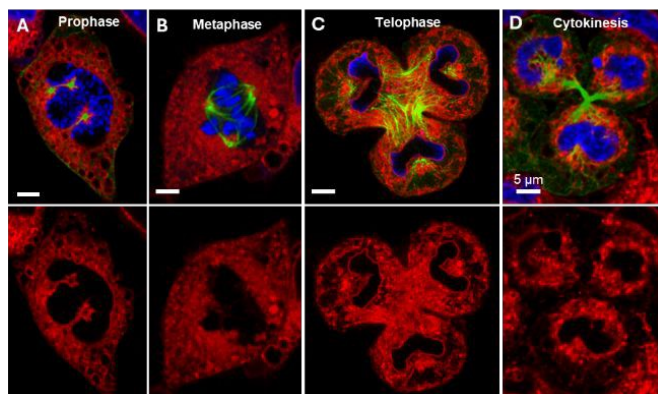


Figure 9. Imaging of tripolar mitosis using NM-ER (red) and CellLight tubulin GFP (green) and Hoechst (blue). Images of tripolar HeLa cells co-registered with DNA (blue), microtubules (green) and lipids (red) in the top panels and the same cells showing NM-ER alone in the bottom panels for (A) prophase, (B) metaphase (C) telophase and (D) cytokinesis. For 3D z-stacks see SI Movies S12. Scale bars 5 μm .

Conclusions

Using non-covalent fluorescence labelling strategies offers exciting opportunities for cell imaging as they reduce fluorophore complexity by eliminating the need for reactive functional groups and are relatively easy to use (Figure 10). However, successful labelling requires partnership with the cell since it controls the intracellular positioning of the fluorophores. Non-covalent approaches have found particular utility for staining plasma membranes through transitory labelling before fluorophore internalization.^{5b,35} They have also found uses for labelling lysosomes in which a fluorophore becomes entrapped within the acidic vesicles, and lipid droplets in which highly lipophilic compounds accumulate.^{36,37} Yet, this approach has seen much less use in the labelling of intracellular organelle membranes of mitochondria, the ER and the NM.^{38,12} As cells produce thousands of different lipid species performing key roles within membrane and organelle structures, signaling, and energy storage it makes their study particularly challenging

as they are not amenable to covalent fluorophore labelling. In this study we expanded the non-covalent labelling to the concept of migratory labelling as the initial structures labelled are not permanent and undergo deconstruction and reconstruction through normal cell division. This places an additional challenge on the fluorophore to remain with the lipid components, without covalent linkage, of the original structures throughout cell division changes. To achieve this aim, the fluorophore NM-ER has been engineered to bypass accumulation in the plasma membrane in favor of the internal membranes of the ER and NM with limited accumulation in lipid droplets. This was achieved through the inclusion of two primary aliphatic alcohol groups on the non-charged fluorophore. The lack of charge inhibits accumulation within the plasma membrane while the alcohol groups offer the potential for hydrogen bonding within intracellular membranes. Purposefully, this accumulation does not exhibit preferential affinity toward any particular lipid species. A contributing factor to NM-ER's migratory abilities may be that as an aliphatic diol it forms hydrogen-bonded networks which connect neighboring lipid head groups within the membrane (Figure 10, red box inset). Such hydrogen bonds are known for aliphatic alcohols and networks have been reported for simpler diols.³⁹ Though it should be noted that the potential for NM-ER redistribution independent of lipid movement does exist. Despite the well-known changes in DNA and microtubular structures during mitosis, systematic monitoring of lipid changes is relatively unexplored. A lipid mapping could provide insights into how their distributions respond to biological stimuli and mechanical forces, during normal and abnormal division events. With the use of NM-ER as a non-covalent migratory lipid marker this can now be achieved with key lipid features observable, including their association with microtubules, chromatin discs and midbody, from this single molecular fluorophore negating the need for cell transfections or antibodies. By using this soft non-invasive approach, we shadow the cells' own machinery that redeploys lipids throughout mitosis as the structures from which they are built disassemble and reassemble. As the NM-ER fluorophore wavelengths are compatible with other common nuclear and GFP stains, it is possible to position lipids spatially and temporally relative to DNA and microtubules. In intracellular lipid research, NM-ER's just-add-and-image ease of use, along with its suitability for time-lapse live or fixed cell imaging, confocal, widefield, or STED microscopy should allow for widespread application.



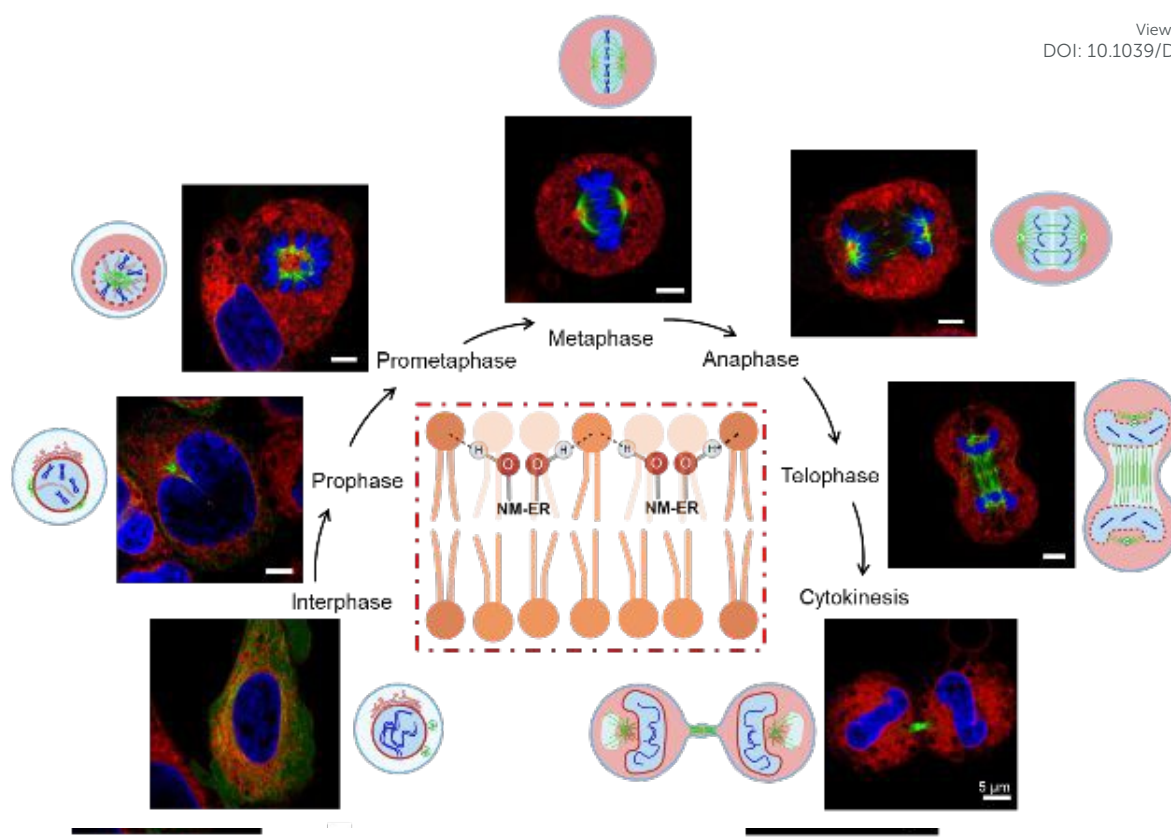


Figure 10. Mitotic mapping of lipids with NM-ER summary including co-registration with DNA and microtubules. Representative fluorescence images of HeLa cells with NM-ER (red), Hoechst (blue) and tubulin-GFP (green) and schematic representative of each cell division phase. For 3D cell views see SI Movies S13. Scale bar 5 μm . For additional experiment images see SI Figure S8. Inset (dashed red box): Potential for hydrogen bonding from aliphatic alcohol functional groups of NM-ER to lipid head groups

METHODS

In solution measurement. 5 μM of NM-ER was diluted in methanol or water (from a high concentration of fluorophore in methanol) or lipid mixtures obtained from a liposome kit (L4395, Sigma-Aldrich). NM-ER synthesized as previously reported.¹²

Cell Culture. HeLa Kyoto, RAW 264.7 and MDA-MB 321 cells were cultured in Dulbecco's Modified Eagles Medium supplemented (DMEM) with 10% fetal bovine serum (FBS), 1% L Glutamine, and Penicillin/Streptomycin (1000 U/mL), and incubated at 37 $^{\circ}\text{C}$ and 5% CO_2 . Cells were seeded on an eight well chamber slide (Ibidi) at a density of 1×10^4 cells per well for 48 to 72h (about 80% confluency).

Image Acquisition. As previously described, images were acquired on a Leica Stellaris 8 Falcon microscope REF. Live cell imaging experiments were carried out at 37 $^{\circ}\text{C}$ and CO_2 at 5%. Images were acquired using a Leica HC PL APO CS2 100X/1.40 oil immersion objective (unless specified) with an excitation at 594 nm for NM-ER and 495 nm for green tubulin obtained by tuning the White Light Laser or a 405 nm laser for excitation of Hoechst (all at about 0.3 μW at the sample). Images were acquired at 200/ 400 Hz with a pixel dwell time of 1.5-2 μs using

a HyD (S or X type) detector collecting emission in counting mod except for Hoechst (HyD S1 detector in analog mode).

As previously described for NM-ER in STED REF, a depletion laser at 775 nm was used at 20% (0.21 mW) with the TauSTED mode from Leica Microsystem and line accumulation of usually 4 lines (2 for z-stacks).

Image processing. All images were processed using Leica LasX software with Lightning post processing applied with three iterations. ImageJ 1.54f was also used to post-process images and Igor Pro 8.04 to plot the graphs.

Live cells experiment. Cells were washed one time with 250 μL of prewarmed media and stained with $\approx 0.4 \mu\text{M}$ of NM-ER for at least 30 min. Same procedure for Hoechst at 3 μM (62249, Thermo Scientific) except for long term experiment where 50 nM was used due to impaired mitosis caused by Hoechst. Tubulin was labelled with a GFP using the CellLight Tubulin-GFP, BacMam 2.0 reagent (C10613, Invitrogen). After cells were seeded for 24h or 48h (confluency of about 60%), the media of a well was removed and replaced by a mixture of 150 μL of media and 25 μL of reagent for another 24h. Cells were then imaged directly and then fixed. For MitoTracker Green (M7514, Invitrogen), the media was replaced with prewarmed media containing 50 nM of the staining and incubated for 30 min as recommended.



ARTICLE

Journal Name

Fixed cells experiment. Cells were washed three times with prewarmed PBS and fixed for 10 min with a prewarmed 4% PFA solution (in PBS). Cells were washed and could be stored at 4°C until needed. Fixed cells were stained with the same concentrations as for live cells experiments. Cells were stained with WGA-Alexa Fluor 647 (W32466, Invitrogen) diluted in PBS to a final concentration of 2 µg / mL overnight at 4°C.

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Conflicts of interest

DFOS has a financial interest in patents granted relating to NIR-fluorophores. All remaining authors declare no conflicts of interest.

Data availability

Data Availability Statement: Data for images contained within the article, supplementary information and additional examples are accessible in the Zenodo database at DOI [10.5281/zenodo.15175573](https://doi.org/10.5281/zenodo.15175573).

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ARTICLE

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Data availability

Data Availability Statement: Data for images contained within the article, supplementary information and additional examples are accessible in the Zenodo database at DOI [10.5281/zenodo.15175573](https://doi.org/10.5281/zenodo.15175573).

