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MPLEx: a method for simultaneous pathogen inactivation and extraction of samples for multi-omics profiling†

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The continued emergence and spread of infectious agents is of great concern, and systems biology approaches to infectious disease research can advance our understanding of host–pathogen relationships and facilitate the development of new therapies and vaccines. Molecular characterization of infectious samples outside of appropriate biosafety containment can take place only subsequent to pathogen inactivation. Herein, we describe a modified Folch extraction using chloroform/methanol that facilitates the molecular characterization of infectious samples by enabling simultaneous pathogen inactivation and extraction of proteins, metabolites, and lipids for subsequent mass spectrometry-based multi-omics measurements. This single-sample metabolite, protein and lipid extraction (MPLEx) method resulted in complete inactivation of clinically important bacterial and viral pathogens with exposed lipid membranes, including *Yersinia pestis*, *Salmonella* Typhimurium, and *Campylobacter jejuni* in pure culture, and

Yersinia pestis, *Campylobacter jejuni*, and West Nile, MERS-CoV, Ebola, and influenza H7N9 viruses in infection studies. In addition, >99% inactivation, which increased with solvent exposure time, was also observed for pathogens without exposed lipid membranes including community-associated methicillin-resistant *Staphylococcus aureus*, *Clostridium difficile* spores and vegetative cells, and adenovirus type 5. The overall pipeline of inactivation and subsequent proteomic, metabolomic, and lipidomic analyses was evaluated using a human epithelial lung cell line infected with wild-type and mutant influenza H7N9 viruses, thereby demonstrating that MPLEx yields biomaterial of sufficient quality for subsequent multi-omics analyses. Based on these experimental results, we believe that MPLEx will facilitate systems biology studies of infectious samples by enabling simultaneous pathogen inactivation and multi-omics measurements from a single specimen with high success for pathogens with exposed lipid membranes.

Infectious disease research is of global interest since the emergence and spread of infectious agents represent ongoing challenges due to population growth and associated increased livestock production to meet food demands, increased urbanization and land-use changes, and greater travel.^{1–5} Emerging infectious diseases are often zoonotic and can be transmitted to humans from animals (e.g., Middle Eastern Respiratory Syndrome coronavirus (MERS-CoV); West Nile virus, influenza A viruses, and Ebola virus).³ As recently demonstrated by the 2014 Ebola virus infection diagnoses in the United States, the ease of world travel and increased global interdependence have added complexity to containing these infectious diseases.⁶ Similarly, as new infectious diseases evolve and emerge, pre-existing infectious diseases re-emerge with new genetic adaptations. For example, novel antigenically distinct subtypes of influenza A viruses that are not recognized by neutralizing

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antibodies can go undetected and cause pandemic outbreaks.⁷ Overuse of antimicrobial drugs and decreased compliance with vaccination policies have led to the development of resistant pathogens (e.g., drug-resistant *Staphylococcus* and *Campylobacter*)^{8,9} and re-emergence of diseases that were previously under control (e.g., pertussis and measles).^{10,11} In addition, uncontrolled neglected tropical diseases such as Dengue fever and West Nile encephalitis that are endemic in developing countries are now emerging in the United States.^{12–14}

In response to these global threats, the National Institute of Allergy and Infectious Diseases (NIAID) Systems Biology for Infectious Disease Research Program supports research focusing on host–pathogen interactions that are characterized using combined multi-omics approaches and dataset integration to “develop and validate predictive models of infectious disease initiation, progression, and outcomes”.¹⁵ Infectious disease research using a systems biology approach is imperative to understanding host–pathogen relationships and allows for development of new therapies and vaccines.^{16–18} Specifically, proteomics, metabolomics, and lipidomics measurements can assist in unraveling host–pathogen relationships. Proteins are the major effectors of cellular pathways and represent the dynamic expression of information encoded within the genome during infection. Metabolites are intermediates and products of cellular pathways and represent the level at which most pharmaceuticals exert their effects.¹⁹ In addition to having key functions in signaling pathways, energy storage and the structural integrity of cell membranes, lipids also function in host–pathogen interactions and immunomodulation since they act in first line recognition and host cell signaling during pathogen docking, invasion and intracellular trafficking.²⁰ However, proteomic, metabolomic, and lipidomic characterizations of infectious biological specimens outside of appropriate biosafety level (BSL) containment laboratories can take place only subsequent to pathogen inactivation.

Non-enveloped viruses such as adenoviruses, noroviruses and bacterial spores are resistant to most disinfectants and require alternative methods for inactivation.^{21,22} Pathogens with exposed lipid membranes are more susceptible to disinfectants including detergents and other solvents.^{23–25} Organic solvents render many pathogens non-infectious by solubilizing and disrupting their lipid membranes or envelopes,^{26–28} and inactivation of pathogens by organic solvents has been leveraged for vaccine development,^{29–32} transfusion fluids,^{33–36} and sanitation.^{37–39} In systems biology studies of pathogenic bacteria and viruses, it would be highly efficient to both inactivate pathogens and extract the molecular components needed for proteomic, metabolomic, and lipidomic analyses in a single step. Two of the most highly cited publications for lipid-based extraction methods, Folch *et al.*⁴⁰ and Dyer *et al.*⁴¹ use high ratios of organic solvents to sample (e.g., 4 : 1), illustrating the broad utility of this technique in cell and tissue extraction. Recently, variations of the Folch technique have had broader utility in the extraction of metabolites and proteins.^{42–45}

Here, we demonstrate that a modified Folch⁴⁰ technique using chloroform/methanol for simultaneous protein, metabolite,

and lipid extraction and subsequent mass spectrometry (MS)-based multi-omics analyses also results in concurrent pathogen inactivation. Specifically, we investigated if the metabolite, protein and lipid extraction (MPLEX) protocol⁴⁶ could inactivate a diversity of infectious agents with exposed and embedded lipid bilayers including three Gram-negative bacteria (*Yersinia pestis*, *Salmonella enterica* subspecies *enterica* serovar Typhimurium, and *Campylobacter jejuni*), Gram-positive community-associated methicillin-resistant *Staphylococcus aureus* (MRSA) isolate USA300, *Clostridium difficile* strain 630 spores and vegetative cells, and four RNA viruses (West Nile [WNV-New York 1999], MERS-CoV [icMERS], Ebola [Ebola-Zaire delta-VP30] and Influenza H7N9 [wild-type, A/Anhui/1/13 and mutant A/Anhui/103F-106M]). We also tested if MPLEX could inactivate adenovirus type 5, which does not contain an outer lipid bilayer.

MPLEX uses chloroform, methanol, and water (8 : 4 : 3) to induce a tri-phasic partitioning of the sample into metabolite, protein, and lipid fractions. Metabolites are located in the

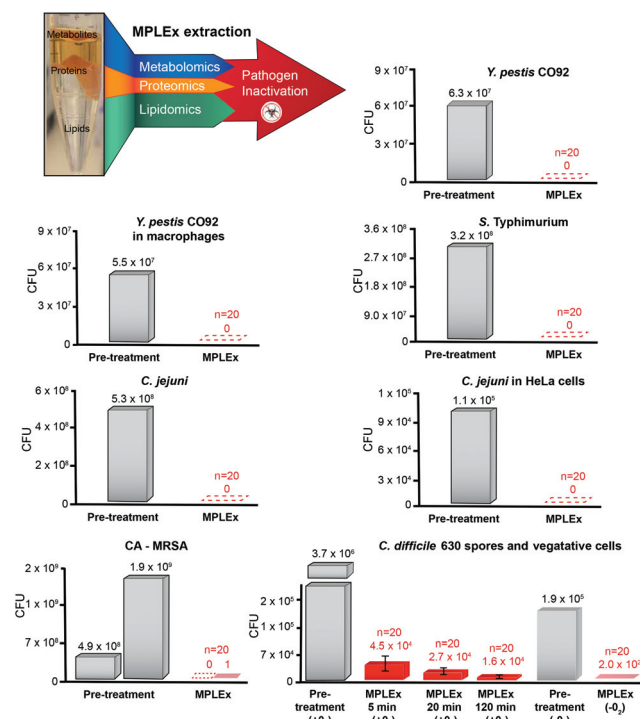


Fig. 1 MPLEX inactivation of bacterial pathogens. (Top, Left) Schematic of the MPLEX extraction method which uses chloroform, methanol, and water (8 : 4 : 3) to induce simultaneous pathogen inactivation and tri-phasic liquid partitioning into metabolite, protein, and lipid fractions. The bargraphs depict the level of pathogen inactivation after MPLEX extraction by quantifying the number of Colony Forming Units (CFU) in pre-treatment control samples (grey) and MPLEX-treated samples (red) in pure cultures of *Yersinia pestis* strain CO92 (*Y. pestis* CO92), *Salmonella enterica* subsp. *enterica* serovar Typhimurium (*S. Typhimurium*), *Campylobacter jejuni* (*C. jejuni*), methicillin-resistant *Staphylococcus aureus* USA300 (CA-MRSA), and *Clostridium difficile* strain 630 (*C. difficile* 630) spores and vegetative cells and in *Y. Pestis* CO92 and *C. jejuni* infection studies in macrophages and HeLa cells, respectively.

upper aqueous layer, whereas lipids are located in the lower organic layer after centrifugation, with a protein disc situated between the two phases (Fig. 1). In this study, MPLEx and subsequent assessment of pathogen inactivation was performed across multiple laboratories, where each laboratory has unique expertise and experience studying the pathogens in question. All studies presented in this manuscript were conducted in accordance with appropriate biosafety guidelines performed in BSL containment laboratories at the University of Wisconsin-Madison, Ohio State University, Washington State University, University of North Carolina at Chapel Hill, University of Texas Medical Branch, and Washington University School of Medicine, all of which are approved for such use by the Centers for Disease Control and Prevention and by the US Department of Agriculture.

The ability of MPLEx to inactivate pathogens was determined by comparing pathogen presence and abundance in either pre-treatment or positive controls *vs.* MPLEx-treated samples. *S. Typhimurium*, *C. jejuni*, MRSA, and *C. difficile* spore inactivation experiments (pre-treatment and MPLEx-treated) were replicated 20 times. *Y. pestis* and viral inactivation experiments were replicated 20 times for MPLEx treatment and a single sample was used for assessment of viral activity in the pre-treatment or positive control. We describe below the basic pathogen inactivation and simultaneous multi-omics extraction protocol. Because methods for culturing bacteria and viruses and for establishing infections vary depending on the model pathogen, we include those relevant experimental details in the description of each study in the ESI.†

MPLEx inactivation protocol

1. In a biosafety cabinet (BSC), remove media from infected cells; immediately wash cells with a suitable buffer. The authors recommend a buffer that can be used to quench cell metabolism, such as a solution maintained at $\sim -40^\circ\text{C}$ and consisting of 60% methanol and 0.85% ammonium bicarbonate in water;⁴⁷ remove rapid quenching solution.

2. Add 150 μL of ice-cold 150 mM ammonium bicarbonate solution; scrape cells off of plates or wells for cells grown on agar or in well plate format. Collect cells and buffer into a 1.5 mL Sorenson microcentrifuge tube (or glass vials); if screw cap tops are required (*e.g.* for BSL-3+ level pathogens) use Fisher Screw Cap microcentrifuge tubes (Cat # 3468).

3. Add 600 μL of MPLEx solution ($\sim -40^\circ\text{C}$ chloroform/methanol [$\text{CHCl}_3/\text{MeOH}$; 2:1, v/v and a 4-fold excess to sample volume]).

4. Vortex for 10 s, leaving the samples on ice for 5 min, and vortex again for 10 s.

5. Remove a 100 μL aliquot of mixed solution for inactivation assay.

6. Centrifuge the remaining solution at 13 000g for 10 min.

7. Remove top phase to fresh tube, remove bottom phase to second tube leaving protein disc behind. Rinse the protein disc with 200 μL methanol and pellet at 9000 g for 5 min.

Decant the methanol solution and allow pellet to dry in BSC for 5 min.

8. All tubes are then removed from BSC and BSL containment laboratories after appropriate disinfection and either stored at -80°C or dried *in vacuo* (for safe shipping of metabolite and lipid phases) and then stored at -80°C .

Some of the bacterial pathogens and all of the viral pathogens were assessed in infection studies where infected host cells were treated with MPLEx and inactivation was evaluated *via* inoculation of the resulting cell lysates into fresh cells. For these infection studies, the MPLEx-treated cell lysates were diluted to retain host cell viability, as undiluted samples can induce toxicity. Some validations of the inactivation procedures were carried out with samples that had been dried in the speed-vac, rather than the liquid/diluted extract prior to drying.

As depicted in Fig. 1 and 2, the MPLEx method for extraction of proteins, metabolites and lipids from a single sample for MS-based multi-omics profiling also simultaneously inactivates a number of clinically important bacterial and viral pathogens with exposed lipid membranes, including *Y. pestis* CO92, *S. Typhimurium*, and *C. jejuni* in pure culture and *Y. pestis* CO92, *C. jejuni*, WNV, MERS-CoV, Ebola and influenza viruses in infection studies. Near complete inactivation ($>99\%$) was observed for pathogens without exposed lipid membranes including CA-MRSA, *C. difficile* 630 spores and vegetative cells after ≥ 20 min MPLEx exposure time, and Ad5 (see ESI and Table S1† for details).

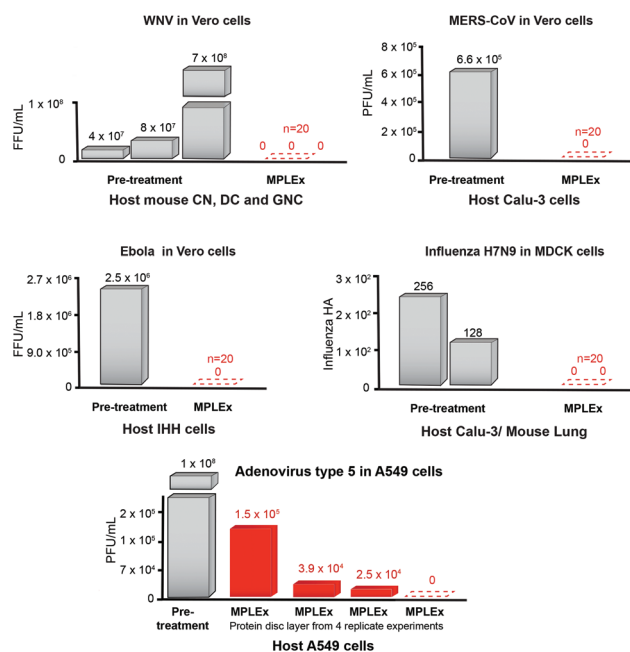


Fig. 2 MPLEx inactivation of viral pathogens. The bargraphs depict the level of pathogen inactivation after MPLEx extraction by quantifying the number of Focus Forming Units (FFU) or Plaque Forming Units (PFU) in pre-treatment control samples (grey) and MPLEx-treated samples (red) in infection studies of West Nile (WNV, New York 1999), MERS-CoV (icMERS), Ebola (Ebola-Zaire delta-VP30), Avian Influenza (H7N9) and Adenovirus type 5 (Ad5).

In addition to demonstrating that MPLEx yields biomaterial of sufficient quality for subsequent multi-omics analyses, Fig. 3 highlights the reproducibility of proteomics, metabolomics and lipidomics data from human epithelial lung cells that were infected with two strains of influenza H7N9

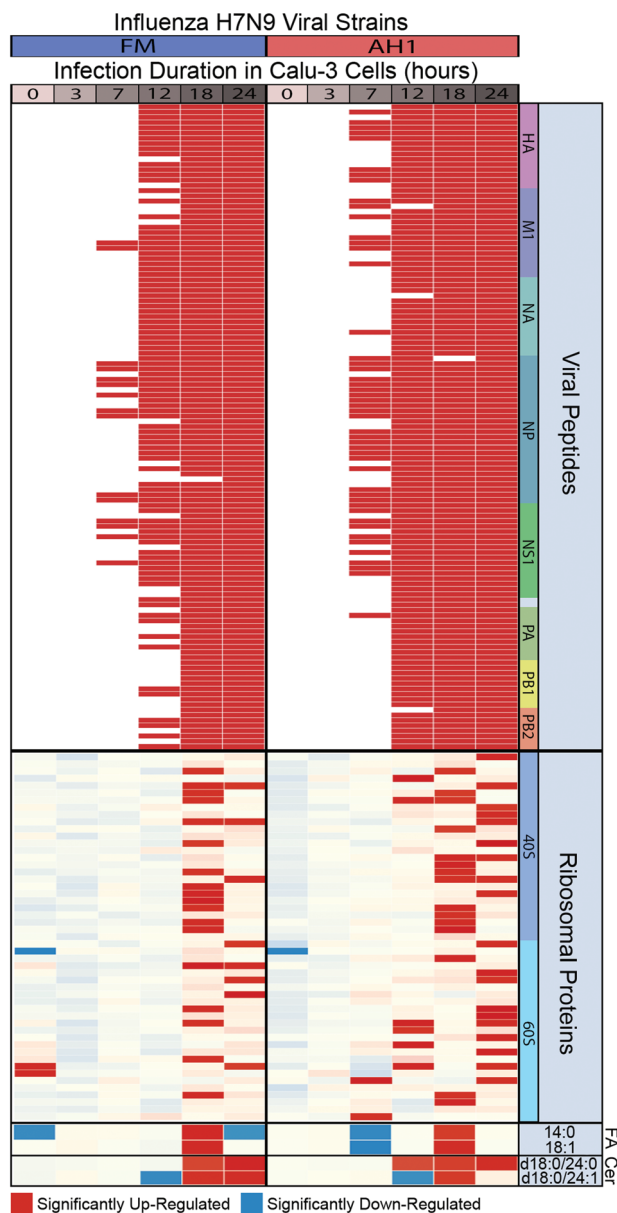


Fig. 3 Reliable multi-omic measurements resulting from MPLEx extraction of influenza infected Calu-3 cells. The heatmap depicts significant abundance changes between Calu-3 cells treated with wild-type (A/Anhui/1/13 [AH1]) and mutant (A/Anhui/103F-106M [FM]) Influenza H7N9 viral strains and time-matched (0, 3, 7, 12, 18 and 24 hours) mock-infected controls. Red color, significant increase, and blue color, significant decrease, in virus vs. time matched mock (p -value < 0.05). The viral peptides labeled at the top of the heatmap include, hemagglutinin (HA), matrix protein 1 (M1), neuraminidase (NA), nucleoprotein (NP), non-structural (NS1), non-structural (NS2) (corresponds to the two unlabeled viral peptides), and polymerase complex proteins (PA, PB1, and PB2); fatty acids, FA; Ceramide lipids, Cer.

(A/Anhui/1/2013), a wild-type (AH1) or a mutant version (FM) which possesses two mutations in the NS1 gene (L103F and I106M) that reduce virus pathogenicity in mice (A. Einfeld, S. Fan and Y. Kawaoka, unpublished results). To demonstrate the utility of the MPLEx protocol for both inactivating pathogens and for obtaining high quality metabolite, protein, and lipid fractions for respective omics analyses, we implemented the protocol in a systems biology study of influenza infection in a human lung epithelial cell line. The MPLEx protocol was implemented to extract proteins, metabolites and lipids from Calu-3 cells infected with AH1 and FM influenza H7N9 virus strains, or mock-infected controls for each of 6 timepoints: 0, 3, 7, 12, 18 and 24 h, and using 5 biological replicates per sample (treatment and timepoint). From this experiment, we quantified 23 688 peptides, 81 metabolites (50 were identified through matching of experimental mass spectra and retention indices to entries in the Agilent Fiehn Metabolomics Retention Time Locked Library),⁴⁸ and 251 and 245 lipids from analyses in negative and positive electrospray mode analyses, respectively; therefore as previously reported⁴⁶ the number of detected analytes with MPLEx provides comparable or more identifications compared to other methods. To assess reproducibility, we calculated the coefficient of variation (CV) of the 5 biological replicates within each of the datatypes (protein, metabolite, and lipid). For proteomics, metabolomics, and lipidomics, the maximum CV across features within each set of biological replicates was < 0.24 (proteomics), < 0.17 (metabolomics), < 0.22 (lipidomics data collected under negative electrospray ionization (ESI)), and < 0.19 (lipidomics data collected under positive ESI) (Table S2†). Fig. 3 shows viral peptides, ribosomal proteins, metabolites and lipids significantly increasing or decreasing in infected samples compared to matched mock controls due to infection duration across both viral strains (Table S2†). Peptide, metabolite, and lipid statistics were performed by comparing data from matched virus and mock-infected controls using Analysis of Variance (ANOVA) with a Dunnett multiple test correction within time point and for qualitative changes *via* a G-test with a Bonferroni multiple test correction within time point.⁴⁹ Peptides, metabolites, and lipids with a p -value less than 0.05 were identified as significantly different (Table S2†). The top of Fig. 3 depicts the 123 viral peptides belonging to viral proteins; hemagglutinin (HA), matrix protein 1 (M1), neuraminidase (NA), nucleoprotein (NP), NS1, NS2, and RNA polymerase complex (PA, PB1, and PB2) significantly increased by G-test (only observed in infected samples) as early as 7 h with the number of significant peptides increasing from 7–24 h. Statistical analysis of our proteomic data also found significant quantitative increases in the relative abundance of ribosomal subunits 40S and 60S with infection duration across both viral strains. During the influenza life cycle, single-stranded viral RNAs migrate to the nucleus where they are copied into mRNA by the viral RNA polymerase complex. Invading viruses do not harbor functional ribosomes in their virions; therefore, viral mRNAs hijack host cell ribosomes and use sophisticated mechanisms⁵⁰ to enable selective translation of viral mRNAs.

Free fatty acid metabolites myristic acid (14:0) and oleic acid (18:1) significantly increased at 18 h post infection across both viral strains (Fig. 3). Ceramides (Cer) containing stearic acid (18:0) and tetracosanoic acid (24:0) significantly increased at 18 and 24 h in both AH1 and FM and at 12 h in AH1; likewise, Cer containing stearic acid (18:0) and nervonic acid (24:1) significantly increased at 18 h in both AH1 and FM and at 24 h in FM (Fig. 3). It has been previously reported that pathogenic avian influenza viruses, such as H7N9, decrease expression of lipid metabolism genes.⁵¹ A NS1 mutation in the FM strain reduces overall pathogenicity; at the early 7 hour time point of infection the wild-type AH1 strain shows over a 30% increase in viral peptide compared to the FM mutant (Fig. 3). Correspondingly at this 7 h time point ribosome expression is only significantly increased in the AH1 virus and at the late 24 hour time point significant ribosomal expression is increased by over 40% in the AH1 compared to FM.

Based on these results we believe that MPLEx will benefit and facilitate systems biology studies of infectious samples by enabling both pathogen inactivation and multi-omics measurements from a single specimen with high success for pathogens with exposed lipid membranes. Fig. 4 and 5 illustrate the bacterial and viral pathogens from this study that contain exposed and embedded lipid membranes; these membranes are denoted with orange color. Of the bacterial pathogens characterized in this study, *Y. pestis* CO92, *S. Typhimurium*, and *C. jejuni* are Gram-negative bacteria, CA-MRSA is a Gram-positive bacterium, and *C. difficile* 630 is a spore forming Gram-positive bacterium (Fig. 4). The exposed walls for Gram-positive bacteria have a higher peptidoglycan (PG) and lower lipid content than Gram-negative bacteria. This bacterial classification is based on the Gram staining method, which differentiates bacteria by the chemical and physical properties of their cell walls; it detects PG, which is present in a thick layer in Gram-positive bacteria.⁵² The inner membrane

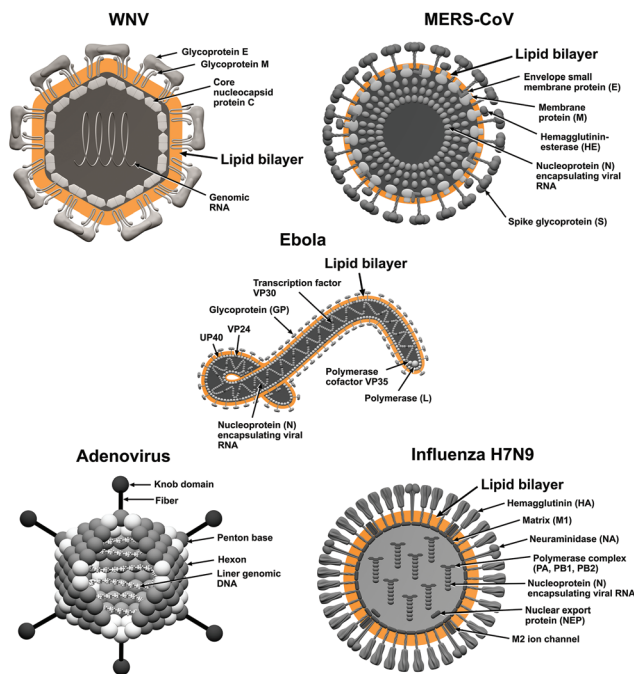


Fig. 5 The structure of lipid enveloped viruses; West Nile Virus (WNV), Middle East Respiratory Syndrome Coronavirus (MERS-CoV), Ebola, Adenovirus and Avian Influenza Virus. Lipid bilayers are denoted with orange color.

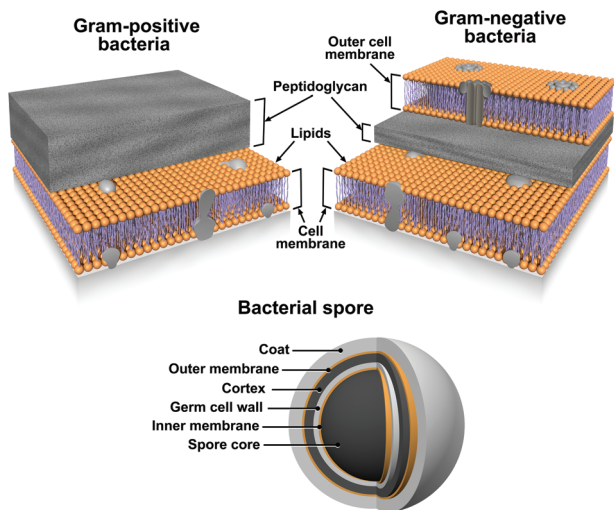


Fig. 4 The structural layers of Gram-positive and -negative bacteria and bacterial spores. Lipid membranes are denoted with orange color.

of dormant spores is composed of a bilayer of immobile phospholipids, which has similar phospholipid composition to growing bacteria but exhibits low permeability to small molecules including water.^{53,54} This inner membrane surrounds the spore core which contains the genome. The basic spore architecture is conserved;^{55–57} outside of the inner membrane is a layer of PG, known as the germ cell wall that is encircled in a thick layer of a modified form of PG, the cortex, which is essential for the acquisition and maintenance of heat resistance. The cortex is surrounded by an outer membrane derived from the mother cell that is essential for spore formation but confers no resistance properties.⁵⁸ A proteinaceous coat surrounds the outer membrane. In *C. difficile* 630 spores, the coat is further enclosed within a structure known as the exosporium. The partial inactivation results presented for CA-MRSA (where only 2 out of 20 experimental replicates still retained a small level of bacterial activity) and *C. difficile* 630 spores reveals that MPLEx can dissolve protective PG layers encircling lipid bilayers and with long enough exposure times could lead to complete pathogen inactivation. The level of inactivation of *C. difficile* 630 spores due to MPLEx treatment exposure time in min was $120 > 20 > 5$ (Fig. 1), in addition in the two replicate experiments of CA-MRSA the experiment with the lower initial bacterial concentration (pre-treatment, 4.9×10^8) did show complete inactivation due to MPLEx. WNV, MERS-CoV, Ebola, and Influenza H7N9 virus all contain lipid bi-layered envelopes (Fig. 5). WNV virions acquire their lipid envelope by budding into the endoplasmic reticulum of the host cell; Coronaviruses such as MERS-CoV acquire their mem-

branes in the ER-Golgi intermediate compartment (ERGIC) and use normal cellular processes to leave the cell; and Ebola and Influenza H7N9 virions acquire their lipid envelopes by budding from host plasma membranes often at lipid raft microdomains that are enriched in sphingomyelin and cholesterol.^{59–61} Ad5 was the only virus that was not completely inactivated by MPLEx; Ad5 is a non-enveloped virus and therefore does not contain any lipid membranes (Fig. 5).

In conclusion, we show that MPLEx completely inactivates pathogens with exposed lipid membranes, such as enveloped viruses and Gram-negative bacteria, thereby facilitating multi-omics measurements from a single specimen in clinically important pathogen studies. Pathogens with internal or protected lipid membranes, such as Gram-positive bacteria and bacterial spores still show a significant decrease in activity (>99% reduction in the number of viable microorganisms after MPLEx treatment) related to both MPLEx solution exposure time and pre-treatment pathogen levels. Even Ad5, a virus without a lipid membrane, showed >99% decreased activity after MPLEx exposure, presumably since non-enveloped viruses can still be susceptible to organic solvent, in particular CHCl₃, due to the denaturation of proteins that are solvent sensitive.^{28,62} In summary, we believe our MPLEx method for concurrent pathogen inactivation and extraction of samples for multi-omics profiling will be broadly applicable to samples containing clinically important bacterial and viral pathogens with exposed lipid membranes since molecular characterization of infectious samples outside of appropriate biosafety containment can take place only subsequent to complete pathogen inactivation. We note that, for biosafety reasons, this protocol should be evaluated in each investigator's laboratory for efficacy in pathogen inactivation, prior to implementation and particularly for pathogens that were not evaluated in our study.

Author contributions

K. E. B.-J. and T. O. M. designed research and wrote the paper; J. E. K., A. J. E., C. P. C., K. G. S., J. F. G., F. H., N. M. N., A. C. S., S. C., L. B. T., P. J. H., K. B. W., Y.-M. K., E. M. Z., C. D. N., K. K. W., B.-J. M. W.-R., E. S. N., B. A., M. E. K., V. M., R. S. B., M. S. D., Y. K., K. M. W., and R. D. S. performed research, participated in design and execution of inactivation assays with pathogens, and wrote and reviewed the paper.

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