Single-cell quantification of the microbiota by flow cytometry: MicFLY

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SUMMARY

The intestinal microbiota regulates multiple host functions, including digestion and

immune development. Our knowledge of the microbiota has been shaped by available

technology that primarily measures relative abundance. However, understanding the

basis of shifts in microbiota composition requires single cell, absolute abundance

measurements. In response to this problem, we developed Microbiota Flow Cytometry

(MicFLY), a single cell technology that directly quantifies and characterizes total

bacterial abundances with species-level resolution in the microbiota. Using MicFLY, we

can identify all major intestinal taxa, discriminate live from dead bacteria, perform single

cell measurements of heterogeneous bacterial mRNA expression and concurrently

quantify Immunoglobulin (Ig) A and G binding to intestinal bacteria. Using longitudinal

species-resolved, quantitative analysis of the preterm infant microbiota, we identify that

E. coli unbound by IgG and IgA associates with the development of necrotizing

enterocolitis. The application of MicFLY single cell technology permits measurement of

the microbiota at a finer scale and with deeper mechanistic understanding of

compositional changes.

INTRODUCTION

The human microbiome affects nearly all aspects of host physiology, and our technology has shaped how we understand these processes. A common method to measure the microbiome is 16S rRNA gene amplicon sequencing (16S-seq), which has allowed us to associate microbial diversity to a broad variety of host phenotypes and disease[1-5]. 16S-seq can be combined with the isolation of antibody bound-bacteria[6-8] to measure relative immunogenicity of microbial members. In recent years, metagenomics has provided greater detail into the microbiome's total gene content[9-11], revealing functional differences between disease states[12]. However, these sequencing-based methods were developed for phylogenetic[13] or functional[9] classification, not the enumeration of different microbial species[14]. A limitation of sequencing is that its profiles are based on relative abundances, which is inadequate for describing population dynamics[15-17]. Absolute abundances are thus essential for interpreting the cause of compositional shifts. However, assays developed for this purpose[18-20] [21-24] are subject to biases from PCR efficiency[25-30] and DNA extraction[27,31-35], potentially leading to distortions in microbial quantification. New technologies to quantify the microbiota with single cell resolution are necessary to identify the mechanisms by which host-microbiota interactions drive disease.

In this study, we present Microbiota Flow Cytometry (MicFLY), a quantitative single cell technology that uses highly multiplexed bacterial probes and fluorescent hairpins to characterize and measure the microbiota. We verify that MicFLY can detect all major representative members of the microbiota at several taxonomic levels from phylum to

species. Additionally, we show MicFLY can distinguish live from dead bacteria and

measure taxon-targeted mRNA, thus linking transcript abundance to bacterial identity.

By incorporating fluorescent antibodies, MicFLY can quantify host antibody binding to

specific gut bacteria. Finally, we apply MicFLY to fecal samples collected from a

longitudinal cohort of preterm infants and find that lack of Immunoglobulin G (IgG) and

Immunoglobulin A (IgA) binding to *Escherichia coli* is associated with the subsequent

development of necrotizing enterocolitis (NEC).

RESULTS

Microbiota Flow Cytometry (MicFLY): a single cell technique for measuring

bacteria.

MicFLY is a quantitative, single cell technology that uses high-dimensional spectral flow

cytometry to measure individual bacteria in the microbiota **(Figure 1A).** Bacteria are first

extracted from stool using centrifugation/density gradients to remove host cells and

fecal debris. Isolated bacteria are stained with an amine-reactive fixable viability dye

and anti-immunoglobulin fluorescent antibodies, which are then cross-linked to the cell

surface in a two-step fixation protocol using bis(sulfosuccinimidyl)suberate (BS3) (a

membrane-impermeable chemical crosslinker), followed by paraformaldehyde (PFA).

Together, these fixatives increase the stability of fluorophore-conjugated antibodies

bound to surface proteins and preserves intracellular architecture[36]. After fixation,

bacteria are enzymatically permeabilized with lysozyme and lysostaphin, facilitating

hybridization with oligonucleotide 'initiator' probes[37,38] that target bacterial 16S and/or

23S ribosomal RNA (rRNA) and can be customized to taxa of interest **(Figure S1A,**

Table S1). Probes targeting rRNA have an initiator sequence on their 3' end that

anneals to a unique pair of self-assembling fluorescent hairpins **(Figure S1B)**. Once

annealed, hairpin pairs undergo a chain reaction (Hybridization Chain Reaction; HCR)

to create a DNA scaffold capable of reaching over 10 kilobases[37-39]. Probe panel

multiplexing is limited only by the number of hairpin-conjugated fluorophores available

(Supplemental Note S1, Table S2). Importantly, by incorporating measurements of

original sample mass with volumetric- or bead-based flow cytometry counting, absolute

abundance can be calculated.

To generate taxon-specific initiator probes, we use both published sequences and those

we designed with a curated rRNA database **(Table S1**, **Data S1).** In contrast to 16S-

seq, MicFLY targets several sites along the entire rRNA molecule, enabling species-

level resolution[40]. MicFLY also supports combinatorial logic with multi-fluorescent

labeling of an individual bacterium at multiple taxonomic levels, massively improving

identification capabilities.

A key advancement of MicFLY is the use of "*Eubacteria*" probes targeting conserved

regions in the 16s rRNA bacterial subunit **(Figure S2A)**. As a proof-of-concept that

Eubacteria probes discriminate bacteria from non-cellular debris, we tested the

'universal' probe (Eub338)[41] on an arabinose-inducible Green Fluorescent Protein

(GFP)-expressing *E. coli* strain[42]. Using GFP as a surrogate for *E. coli* presence, we

demonstrated that virtually all GFP+ events were also stained with Eub338 **(Figure**

S2B). qPCR of GFP+ Eub338+ and GFP- Eub338- sorted fractions revealed *E. coli*

ompX and 16S genes were undetectable in the Eub338- fraction, indicating false

negatives are not a significant issue for MicFLY (**Figure S2B)**. Eub338- GFP- events

also exhibit reduced light scatter properties **(Figure S2C)**, consistent with small particle

debris[43,44]. As expected for two probes binding the same molecule[37], comparison of

Eub338 to a separate *Enterobacteriaceae* probe revealed a linear relationship **(Figure**

S2D). While Eub338 is considered a universal bacterial marker, we designed two

additional pan-bacterial initiator probes (Eub841 and Eub1491) to minimize the chance

of undetected bacteria from the failure of any one probe **(Table S1, Data S2)**[45].

Comparing these two novel probes reveal overlapping signal with Eub338 in mouse

microbiota **(Figure S2E)**; critically, none of the probes bind to fecal matter from germ-

free mouse stool, indicating lack of cross-reactivity with eukaryotic rRNA.

To test if MicFLY maintains cellular morphology, we stained and imaged mixed cultures

of *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive)

(Figure 1B). Confocal imaging revealed uniform morphology (*E. coli* = rods; *S. aureus* =

cocci) and no 'double' stained bacteria, demonstrating that MicFLY is capable of

measuring intact bacteria at the single cell level.

MicFLY measures all major representative members of the intestinal microbiota at

multiple taxonomic levels.

We validated MicFLY can identify individual taxa in a mixed population using *in vitro*

cultured *E. coli*, *S. aureus* and *Bifidobacterium breve*. Critically, MicFLY provided

'digital' identification of all three taxa, as each was positive only for their own probe

(Figure 1C). To demonstrate MicFLY can differentiate between species, we designed

probes targeting *Klebsiella pneumoniae* or *Klebsiella oxytoca*. Species-specific probes

showed extraordinary specificity with virtually zero cross-reactivity **(Figure 1D**),

demonstrating MicFLY can perform precise discrimination of closely related bacteria.

We designed MicFLY such that the fixation and permeabilization enable detection of

bacteria with distinct cellular architecture. To test whether MicFLY was comprehensive

to the most common bacteria in the human microbiota, we constructed a panel of ten

initiator probes **(Table S1)** and assayed fixed cultures of 20 diverse species

encompassing the dominant members of the human microbiota. MicFLY was able to

identify each taxon with high specificity and minimal off-target signal **(Figure 1E)**,

highlighting that MicFLY is effective on a broad variety of bacteria.

MicFLY quantifies the number of live bacteria.

Flow cytometry has been used to measure total bacterial abundance[46-50], typically using

nucleic acid stains that are not exclusive to bacteria and lack taxon specificity. To

establish MicFLY's capacity for bacterial quantification, we serially diluted four bacteria

(*E. coli*, *K. pneumoniae*, *E. faecalis*, and *S. aureus*) and compared abundances in

parallel across three methods: 1) MicFLY; 2) DNA staining (SytoBC), and 3) colony

formation **(Figure S3A).** Using colony-forming units (CFU) as the 'gold standard'

comparison, MicFLY quantification was superior to nucleic acid staining, showing a

linear relationship over a wider range (~10^4-10^8 counts/mL for MicFLY vs. ~10^6-10^8

counts/mL for SytoBC) and closer adherence to CFU for all four taxa, as calculated by

cumulative perpendicular distance **(Figure S3A).** We also evaluated MicFLY's ability to

enumerate bacteria from preterm stool **(Figure S3B)**. Though CFU counts cannot be

performed for fecal samples, we found MicFLY was comparable to Syto BC between

~10^4-10^7 counts/mL **(Figure S3C)**. No correlation was observed between MicFLY-

calculated total abundance and input stool, revealing mass is a poor surrogate in such

samples for bacterial content **(Figure S3D)**.

Current microbiota analysis methods are inefficient for distinguishing viable bacteria[51,52],

hindering our ability to study the important but distinct roles that live and dead bacteria

play in host physiology[53-59]. To address this, we tested an amine-reactive fixable viability

dye in our MicFLY protocol and showed clear discrimination of viable bacteria in heat-

killed bacterial culture (**Figure 1F**) and mouse stool (**Figure 1G**). Overall, MicFLY

functions well for single cell quantification of the live microbiota.

Validation of MicFLY *in vivo* with a known bacterial community.

We next validated MicFLY for microbiota identification and quantification using

gnotobiotic mice colonized with a predefined 9-member community of bacteria derived

from young mice (Pediatric Community; PedsCom)[60,61] **(Figure 2A)** and compared

relative/absolute abundances between MicFLY, qPCR and 16S-seq**.** To have

comparable taxonomic resolution to 16S-seq, we generated family-level probes (7

different families) and validated each on pure culture isolates of each bacterial member

(Figure S4A). Relative abundance measurements showed high concordance between

MicFLY and 16S-seq **(Figures 2B and 2C)**. Accordingly, Bray-Curtis beta-diversity

distance analysis confirmed that relative composition measured by MicFLY and 16S-

seq was more similar to each other than to qPCR (**Figure 2C**). Absolute abundance

counts demonstrated that total numbers for the majority of bacteria were comparable

between MicFLY and qPCR, except for the *Lactobacillaceae* which MicFLY counted as

significantly higher **(Figure 2D).** Enhanced detection of this family was also reproduced

in a smaller gnotobiotic community (PedsCom-6)[61] (**Figure S4B**). Bacterial abundances

calculated by MicFLY were not artificially increased by background auto-fluorescent

events, as germ-free mouse stool established that these events (~10^1-10^3 cells/mg

stool) are too rare to have an impact on abundance measurements **(Figure S4C).**

For these comparative abundance analyses, we measured total *Eubacteria*+ cells by

MicFLY (both live and dead) to maintain consistency with bulk DNA tests **(Figures 2A-**

2D). We were also interested in whether different taxa were over- or under-represented

by live or dead cells. We quantified the live microbiota **(Figure S4D)** and uncovered that

the majority of taxa had similar viability (~80%), except for *Lachnospiraceae* and

Staphylococacceae which were significantly less viable **(Figure 2E).** Re-analysis of

absolute abundance data focusing on live bacteria revealed that qPCR overestimated

the number of viable *Lachnospiraceae* **(Figure S4E)**. These findings uncover how bulk

cell analyses may be substantially affected by dead bacteria in some microbial

communities, which can be circumvented using MicFLY which provides single cell

live/dead discrimination.

MicFly measures bacterial mRNA with single cell resolution.

MicFLY uses HCR, which is effective for detecting bacterial gene expression[37,62-69], but

has not been used to measure taxon-targeted mRNA in the microbiota. To measure

mRNA with MicFLY, we used a split-initiator design in which two initiator probes must

anneal to their target at adjacent sites for fluorescent hairpins to unfold **(Figure**

S1B)[37,70]. We targeted multiple sites along the mRNA to maximize fluorescent signal

(Table S3). To assess whether MicFLY preparation conditions maintain detectable

levels of mRNA, we tested *E. coli* transformed with a plasmid containing GFP controlled

by a pBAD arabinose-inducible promoter[42,71,72] **(Figure 3A)**. Immediately post-induction,

MicFLY measured a rapid rise in both GFP mRNA and protein **(Figures S5A and S5B).**

After four hours, GFP mRNA dropped while reporter protein remained high. This

reduction in mRNA was not due to arabinose depletion as 'spent media' could induce

GFP from unstimulated *E. coli* **(Figure S5C)**. These results are consistent with negative

feedback occurring in the pBAD system due to self-regulation from arabinose

metabolism products[73-75]**.**

To assess MicFLY's sensitivity to different mRNA levels, we tested varying arabinose

concentrations for induction **(Figures 3B and S5D)**. Messenger RNA levels were more

sensitive to arabinose concentration than GFP protein, likely because of the short half-

life of prokaryotic mRNA[76]. To track kinetic differences in mRNA expression, we added

and removed arabinose at different time points and compared transcript level to that

with a constant arabinose supply. MicFLY could identify heterogeneous subpopulations

that emerged at different intervals **(Figure S5E)** and measure a dynamic transcriptional

response **(Figure 3C).** These findings are consistent with heterogeneous on-switching

of the pBAD promoter, which results in both induced and non-induced populations at

any given time[73,77,78], captured here at the single cell level.

Messenger RNA levels produced by high-copy plasmids can be artificially elevated.

Thus, it was important to test whether MicFLY could also measure mRNA expressed

from the chromosome. To test this idea, we measured a constitutively-expressed,

genomically-encoded mCherry mRNA and protein in a strain of *Salmonella enterica*

serovar Typhimurium (*Stm*)[79] **(Figure 3D).** Here we observed that mCherry protein was

consistently high while mRNA was expressed bimodally, indicating heterogeneity in

mRNA levels. Collectively, these analyses demonstrate MicFLY resolves mRNA

transcript levels in single cells *in vitro* and thus can identify heterogeneous

subpopulations with asynchronous gene expression.

Being able to concurrently identify and measure mRNA from individual taxa in mixed

populations would be a major advancement. To determine whether MicFLY could

perform this function, we measured mRNA expression of the *Stm* outer membrane

protein (ompA) and confirmed its detection in *Stm* in a mixed population with *E. coli*

(Figure 3E). As the ompA gene is present in both *Stm* and *E. coli* and only differs by

~10% nucleotide identity **(Figure S5F)**, this result demonstrates the accuracy and

precision of MicFLY in discriminating specific mRNAs. We next measured ompA

expression *in vivo* using a common intestinal infection model of *Stm*[80,81] **(Figure 3F)**.

We identified *Stm* and *Bacilli* as major constituents in the cecum and colon **(Figure 3G),**

and found that ompA expression levels in *Stm* increase significantly at later time points

of infection, but is not detected in *Bacilli* **(Figure 3H)**. These data demonstrate that

MicFLY can perform quantitative, targeted, single-cell transcriptomics in the gut

microbiota.

MicFLY quantifies taxonomically resolved IgA-bound single bacterial cells.

We designed the MicFLY fixation and permeabilization protocol to maintain the structure

of proteins, such as host antibodies, on the bacterial surface. To test this, we incubated

E. coli with breast milk IgA according to our published protocol[82]. Comparison of IgA-

bound fixed bacteria before and after MicFLY showed no difference in binding (**Figure**

S6A). To assess the specificity of antibody binding in MicFLY, we analyzed bacteria

extracted from the stool of wildtype (C57BL/6) or IgA$^{-/-}$ mice and observed virtually no

IgA staining in IgA$^{-/-}$ samples (**Figures S6B and S6C**). In wildtype mice, IgA binding to

various taxa was not uniform, as we found that the *Lactobacillaceae* were highly IgA-

bound compared to other bacteria **(Figure S6C)**. Thus, MicFLY now makes possible the

simultaneous identification and quantification of IgA-bound taxa at the single cell level

from mixed populations collected *in vivo*.

MicFLY identifies altered IgA binding patterns in donor milk-fed preterm infants.

Preterm infants are colonized with an atypical microbiota dominated by facultative

anaerobic bacteria (*Enterobacteriaceae*, *Bacilli*), which is associated with increased

incidence of diseases like NEC[83]. Development of the infant microbiota (including

preterm) is regulated by feeding modality with bioactive molecules in milk (IgA,

oligosaccharides etc.) as potent mediators[84]. Human milk is associated with reduced

incidence of NEC[85], and when the mother's own milk (MOM) is not available, donated

human milk is often used as a substitute. Donor milk (DM) must be pasteurized to

reduce the risk of infection, but pasteurization reduces the amount[86-88] and anti-bacterial

reactivity of IgA[82]. How much milk-derived IgA is required to saturate the infant

microbiota, and whether DM leads to reduced IgA binding of intestinal bacteria, remains

unknown. To test this, we used MicFLY to analyze fecal samples from a longitudinal

prospective cohort study of preterm infants born between 24 and 33 gestational weeks

of age, with stool collection starting at day of life (DOL) 10 **(Figure 4A).** We selected

samples from infants that i) were not treated with antibiotics after DOL 7, ii) did not

develop NEC or other infection-related illnesses, and iii) were either fed exclusively

MOM (n = 6 infants, 29 samples) or exclusively DM (n = 12 infants, 44 samples).

Because infants begin making their own antibodies around three to four weeks post-

delivery[1,89], we limited analyses to DOL 21 to assess the binding of milk-derived IgA to

the microbiota**.** We measured the two taxa (class: *Bacilli* and family:

Enterobacteriaceae) comprising the majority of bacteria in neonatal preterm infants

(Figure 4B)[21,90-92]. Total abundance quantification by MicFLY revealed similar bacterial

loads between DM and MOM-fed infants, averaged across DOL 10-21 **(Figure 4C).**

Despite the lack of differences in total numbers, aggregate data revealed a significant

decrease in the proportion of IgA-bound *Bacilli* and *Enterobacteriaceae* in DM-fed

infants **(Figure 4A)**. ELISA measurement of fecal waters confirmed decreased

concentration of free IgA in DM-fed infants, supporting that pasteurization of milk

reduces infant intestinal IgA levels significantly **(Figure 4E).** We next asked if variations

in bacterial density could be affecting intestinal IgA binding. Interestingly, we found that

Enterobacteriaceae bacterial load had no effect on IgA binding in MOM-fed infants, but

higher abundance was correlated with decreased binding in DM-fed **(Figure 4F)**. These

data indicate that saturation of anti-*Enterobacteriaceae* antibodies may be reached

sooner in DM due to reduced concentrations or reactivity[82]. In contrast, for *Bacilli*,

increased load significantly correlated with decreased IgA binding in both groups,

indicating reactivity differences may also play a role in determining the saturation point

for milk-derived antibodies. Together, these findings reveal that alterations in IgA levels

due to pasteurization alter antibody binding of bacteria in the intestine of infants fed DM.

MicFLY identifies that a lack of IgG and IgA binding to *E. coli* is associated with

the development of necrotizing enterocolitis.

Preterm infants are uniquely at risk for necrotizing enterocolitis (NEC), a deadly

intestinal disease with a mortality rate up to 35%[93,94]. NEC is a multifactorial condition

culminating in acute intestinal inflammation and necrosis. *Enterobacteriaceae* family

bacteria are associated with NEC[1,83]; however, no single species has been identified as

the sole cause[90,95]. Breast milk IgA reduces NEC risk[85], and decreased frequencies of

IgA-bound *Enterobacteriaceae* are associated with NEC development[1]. It is not known if

this loss in antibody binding over time is due to expansion of certain species not

targeted by IgA or whether bacterial overabundance is saturating maternal antibodies in

the intestine. Additionally, maternal IgG is becoming increasingly recognized as critical

to neonatal immunity and may cooperate with IgA during early life to limit gut bacteria-

induced inflammation[96-98]. What role this plays in the preterm microbiota has not been

investigated.

To test MicFLY's ability to quantify the antibody-bound preterm microbiota, we selected

longitudinal samples from a small cohort of preterm infants that either did or did not

develop NEC. We designed a set of probes enabling detection of the most common

taxa found in preterm infants (Class *Bacilli*: *Staphylococcus spp.*, *Enterococcus spp.*,

and *Streptococcus spp.*; Family *Enterobacteriaceae*: *E. coli*, *K. pneumoniae*, *K.*

aerogenes, *K. oxytoca*, *E. cloacae*) **(Figure S7A)**[21,83,90,99-101]**.** This panel permits us to

concurrently measure IgG and IgA binding to each taxon **(Figure S7B).** We collected

fecal samples from patients with prospective NEC diagnosis (n = 7 infants, 72 samples)

or matched control infants who did not develop NEC (n = 6 infants, 71 samples) **(Figure**

5A). Each control infant had at least 1 NEC match based on biological sex, gestational

age at birth (+/- 2 weeks), delivery mode (vaginal or C-section) and feeding

(MOM/partial MOM or DM exclusively) **(Table S4).** NEC samples were collected prior to

diagnosis.

We next used MicFLY to perform quantitative species-level measurements of the

microbiota. Aggregated abundance data showed that among the *Enterobacteriaceae*, *E.*

coli had the highest load in patients who progressed to NEC and was significantly

elevated compared to controls **(Figure 5B)**. For *Bacilli* bacteria, the *Staphylococcus*

species were the most abundant but did not differ between NEC and control. Low

abundance taxa *E. cloacae, Enterococcus* and *Streptococcus* were also higher in NEC,

while *K. aerogenes* was enriched in controls. However, we are cautious of drawing

conclusions from low abundance taxa at or below the assay's limit of detection **(Figure**

S4C).

We next tested if we could use longitudinal data to associate NEC development to

expansion of *Enterobacteriaceae* or *Bacilli* populations. However, supporting previous

reports[102,103], we did not see a correlation between shifts in bacterial load and NEC

(Figure 5C). To comprehensively visualize our single cell data, we graphed our data as

Uniform Manifold Approximations and Projections (UMAPs). UMAP analysis from

aggregated data confirmed total abundance findings **(Figure 5B)** that *E. coli* is

overrepresented in NEC **(Figure 5D)**. In contrast, UMAP analysis of *Bacilli* species

produced highly similar patterns for both groups **(Figure 5E).** Day-by-day projections

made from each patient **(Figures S8 and S9)** revealed that many NEC-affected infants

(Patients 46, 77, 104 and 106), but not controls, are colonized by persistent and/or

increasing populations of *Enterobacteriaceae* and in particular, *E. coli*. These results are

not distortions produced by UMAP visualization as species-level total abundances

calculated for each patient revealed a similar pattern **(Figure S10)**.

We next incorporated antibody binding data into our analyses. UMAPs revealed that

clustering of *Enterobacteriaceae* species **(Figure 5F)**, but not *Bacilli* **(Figure 5G)***,* was

driven by antibody binding intensity, with several *Enterobacteriaceae* species having

high IgA/IgG binding localized to a center cluster **(Figure 5F)**. The proportions of

antibody-bound and unbound subpopulations also differed between NEC and control

(Figure 5F), with more IgA/IgG unbound *E.coli* enriched in NEC. Conversely, IgA/IgG

unbound *K. pneumoniae* was enriched in the control, but this appeared to be driven by

a single patient **(Figure S9).** Quantitative analysis of aggregated longitudinal data

revealed significantly reduced IgA and IgG-bound *Enterobacteriaceae* and IgA-bound

Bacilli in patients who progress to NEC **(Figures S11A and S11B)**, thereby confirming

UMAP analyses. IgG also appeared targeted toward the *Enterobacteriaceae*, as there

was no significant difference between IgG-bound *Bacilli* between NEC or control

(Figure S11B). We asked whether these differences in antibody binding could be due to

increased bacteria exceeding intestinal antibody supply, but we observed no significant

correlation between higher load and lower binding **(Figure S11C)**. Taken together,

these results strongly indicate that deficits in antibody reactivity, not bacterial

overabundance, are contributing to observed differences in binding.

To better understand how antibody binding and species-level abundance dynamics

might differentiate NEC and control, we utilized a multi-modal dimensionality reduction

tool, Joint Compositional Tensor Factorization (Joint-CTF), that takes into account

repeated measurements and sparsity in multi-modal microbiome data to identify

differentially abundant bacteria throughout the microbiota's development[104]. Based on

our analyses indicating that differences amongst *Enterobacteriaceae* discriminate NEC

from control (**Figures 5B, 5D-5G**), we focused on species within that taxon. Joint-CTF

analysis showed clear clustering of patients along the third axis of variation (PC3)

(Figure S11D), which significantly separated them by diagnosis **(Figure S11E)** and

consistently differentiated the two groups over time **(Figure S11F).** The features

contributing to the subject-level separation shown in PC3 (i.e., feature loadings) were

sorted based on their coefficients, with highest rankings most associated to NEC and

lowest rankings associated to control **(Figure S11G)**. The top features associated with

NEC were IgA⁻/IgG⁻ bacterial taxa, specifically *E. cloacae, E. coli* and *K. aerogenes*

(**Figure 5H**). In contrast, IgA⁺/IgG⁺ abundance for many of these same taxa, including

E. coli, was associated with control patients (**Figure 5H**).

As confirmation of Joint-CTF, longitudinal correlation analyses of both percent and

number of IgA/IgG bound bacteria confirmed nearly undetectable IgA⁺IgG⁺ double-

bound *Enterobacteriaceae*, and specifically *E. coli*, in infants who will develop NEC

(Figures 5I, 5J, S11H). Consistent with IgA⁺IgG⁺ data, the percentage of IgA⁻IgG⁻

Enterobacteriaceae and *E. coli* significantly decreases over time in control but not NEC

(Figures 5I and S11H). The absolute number of IgA⁻IgG⁻ *Enterobacteriaceae* and *E.*

coli was also maintained or increasing in some NEC patients but not controls **(Figures**

5J and S11H). Interestingly, IgG⁺ bacteria was almost always exclusively IgA⁺, while

the converse was not true **(Figures 5I, S7B, and S11H)**. These data indicated that lack

of IgG binding to *E. coli* was the dominant association with NEC development. The top

ranked feature, *E. cloacae* **(Figure 5H)**, had too little bacteria to draw meaningful

conclusions **(Figure S11I).**

Taken together, our quantitative analyses of the preterm microbiota highlights the

extraordinary potential of MicFLY to identify factors influencing microbiota dynamics and

proper establishment of the infant gut microbiome. While this cohort is too small to draw

actionable conclusions in regard to NEC, we believe our data demonstrates the striking

ability of MicFLY to quantify the number and properties of individual species in mixed

communities and associate them to disease.

DISCUSSION

Here we demonstrate MicFLY as a single cell technology that performs absolute

quantification of the microbiota using spectral flow cytometry. Various methods have

previously been used to quantify the microbiome, which associate relative abundances

to total abundances with qPCR[18-20] or spiked-in sequences[21-24]. However, confounding

factors may limit accuracy in these approaches. For instance, loss of signal from low

abundance bacteria is common in samples with low biomass, making comparisons

between samples unreliable[105,106]. Relative abundances may also be skewed by

amplification biases caused by unequal primer/template concentration[25-30,107,108] or DNA

isolation efficiencies[27,31-35]. The number of 16S gene copies also varies by species,

causing detection bias toward taxa with higher copy numbers[109,110]. Thus, microbiota

abundances have been difficult to compare across laboratories, limiting accurate

assessment of the role of differential bacterial abundance in the host[35,111]. MicFly

advances beyond these previous methods by enumerating individual bacteria with

spectral flow cytometry, thus eliminating sequencing biases. However, because MicFLY

is a targeted method, sequencing will still be necessary to uncover unknown

microbiotas.

We demonstrate through incorporation of an amine-reactive dye that MicFLY can

measure microbiota viability. Most microbiota sequencing methods do not take into

account bacterial viability because it is challenging to assess[51,112]. Thus, the impact of

bacterial cell death on the gut microbiota and host immunity is not well understood[53-58].

For example, we know that colonization shifts significantly according to circadian

rhythms, but the patterns of bacterial cell life and death are unclear[113-115]. MicFLY's

ability to detect significant quantifiable differences in viability among taxa in the

microbiota opens up further investigation into these questions.

Recently, single cell bacterial transcriptomics have been demonstrated on a variety of

platforms[116-125]. Unlike eukaryotic single cell RNA-sequencing (scRNA-seq), all bacterial

scRNA-seq platforms require extensive pre-processing of the mRNA that potentially

introduce bias. In contrast, MicFLY performs single cell mRNA quantification without

enzymatic amplification of nucleic acids, permitting direct assessment of transcripts. A

substantial breakthrough of MicFLY is the combination of mRNA- and taxon-targeted

probes to measure bacteria-specific gene expression within mixed populations. This

aspect of MicFLY has a plethora of applications, such as investigating how

heterogeneous bacterial populations drive host responses during infection and chronic

inflammatory disease.

By incorporating surface protein-targeted fluorescent antibodies, we demonstrate

MicFLY can quantify the antibody-bound microbiota. Previously, antibody-bound

bacteria could only be studied using Ig-Seq, a method requiring comparison of sorted

populations that is limited to non-quantitative, compositional analyses. Ig-Seq can only

determine whether an antibody-bound population is enriched with a given taxon, but not

how much each taxon is directly bound by antibodies[6]. In contrast, MicFLY directly

measures binding at the single cell level with concurrent IgA/IgG measurements,

revealing independent and overlapping antibody-bound bacteria, as well as preferential

antibody targeting at the species-level. MicFLY also improves on Ig-Seq in that it

functions robustly in low biomass samples (such as preterm infant fecal samples),

whereas species level discrimination with Ig-Seq requires much more material[11].

To demonstrate the effectiveness of MicFLY, we analyzed stool samples from a

prospective longitudinal cohort of preterm infants that did or did not develop NEC.

Critically, we demonstrated that eight individual taxa from two families could be

quantified at the genus/species level alongside IgA and IgG binding. Even in this small

cohort, MicFLY analysis provides highly granular information on longitudinal shifts in the

microbiota and their relationship to disease. We previously identified that infants who

develop NEC were enriched in IgA unbound bacteria[1], but here, using better

technology, we show an additional important role for IgG. Indeed, even in aggregated

longitudinal data, IgG binding of *Enterobacteriaceae* is distinctly enriched in control

patients, which we speculate are controlling pro-inflammatory bacteria during early life[96-

98]. We hypothesize that a larger cohort could elucidate how IgG and IgA may serve

different and/or complementary functions influencing the development of the preterm

infant microbiota in both health and disease.

A potential confounder to our NEC cohort data is the presence of DM-fed infants, which

we demonstrate have much less IgA bound to intestinal bacteria before DOL 21.

However, we would point out that samples from all DM-fed infants in our NEC study (2/7

of NEC and 1/6 of control) were collected for over 50 days, but NEC-affected infants are

dominated by IgG/IgA unbound bacteria long after they should be producing their own

antibodies (around 3-4 weeks of life)[1,89]. These findings beg the question of why only

some infants fail to produce sufficient amounts of microbiota-reactive antibodies.

Another confounder is that four infants in the NEC cohort, but none in the control,

received antibiotics after DOL 7. However, antibiotics were given either far before

(patients 104 and 105) or just before (patient 77) diagnosis, thus, we think is not critical

to the associations identified by MicFLY. A fourth infant (patient 47) also provides just

three samples, which is of limited value overall. Despite these limitations, our work still

displays the incredibly high resolution analyses that MicFLY can provide to elucidate

microbial signatures of disease; a larger cohort would therefore be needed to precisely

define colonization dynamics of the NEC microbiota.

Taken together, MicFLY has the potential to revolutionize microbiome research by

enabling quantitative single cell analysis of mixed bacterial populations without the need

for sequencing or culturing. MicFLY is a highly versatile technology that provides

granular information on bacterial identity, viability, transcription and antibody binding. As

such, MicFLY holds extraordinary promise as a tool for future research requiring highly

dimensional quantitative data to uncover the dynamics driving microbiome-related

disease.

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AUTHOR CONTRIBUTIONS

Conceptualization, C.M.T., D.A.A., W.H.D., T.W.H.; Data curation, C.M.T.; Formal analysis, C.M.T., B.C.V.; Funding acquisition, C.M.T., M.A.S., L.S., W.H.D., T.W.H.; Investigation, C.M.T., A.R.L., T.C.T., I.A.V., S.W., V.L., K.J.S.; Methodology, C.M.T., B.C., D.A.A., M.A.S., L.H., W.H.D., T.W.H.; Project administration, C.M.T., M.B.,

T.W.H.; Resources, M.B., M.A.S., L.S., W.H.D., T.W.H.; Supervision, C.M.T., M.A.S.,

L.S., T.W.H.; Validation, C.M.T., B.C.V., A.R.L, T.C.T., I.A.V., S.W., V.L.; Visualization,

C.M.T., B.C.V.; Writing – original draft, C.M.T., B.C.V., M.A.S., L.H., W.H.D., T.W.H.;

Writing – review & editing, C.M.T., B.C.V., M.A.S., L.H., W.H.D., T.W.H.

DECLARATION OF INTERESTS

TWH, CMT, DAA and WHD have patents on the MicFLY technology in this manuscript.

TWH previously consulted for Keller Postman LLC.

RESOURCE AVAILABILITY

Lead contact

Requests for further information and resources should be directed to and will be fulfilled

by the lead contact, Dr. Timothy Hand (timothy.hand@chp.edu).

Materials availability

All unique/stable reagents generated in this study are available from the lead contact

with a completed materials transfer agreement.

Data and code availability

• Raw data generated from 16S rRNA gene amplicon sequencing for PedsCom

dataset in this study are available as FASTQ files and have been deposited to

FigShare (https://figshare.com/s/93ad90f14bf95e046f26).

• This paper does not report original code.

• Any additional information required to reanalyze the data reported in this paper is

available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Preterm infant fecal samples

The human study protocol was approved by the Institutional Review Board

(STUDY19110318) of the University of Pittsburgh. This work was conducted as part of a

prospective longitudinal cohort study of preterm infants hospitalized in the NICU at

UPMC Magee-Women's Hospital. Written informed consent was obtained from

parents/guardians prior to enrollment. Enrolled infants were born between 24 and 33

weeks gestational age with no other exclusions. Fecal sample collection began at day of

life (DOL) 10 and ended within 63 days post-delivery or when the infant reaches 33

weeks gestational age, whichever comes first. Sample collection stops when infant is

diagnosed with NEC. Fecal samples were collected from the diaper and immediately

frozen at –80°C. Samples from all infants who developed NEC were included in this

study regardless of antibiotic status due to the limited number of diagnosed patients.

Except for infants who developed NEC, samples selected for testing were only from

infants who did not develop any other bacteria-related illness and did not receive any

antibiotics after DOL 7.

Mice

C57BL/6 and 129X1/SvJ mice were purchased from Jackson Laboratories (Bar Harbor,

ME). $Igha^{-/-}$ mice were obtained from Yasmine Belkaid (NIH, Bethesda, MD). Non-

gnotobiotic mice were maintained under specific pathogen-free conditions. Germ-free

C57BL/6 mice were maintained at University of Pittsburgh Gnotobiotic Core facility.

Germ-free and gnotobiotic Eα16/NOD PedsCom colonized mice were maintained at the

Hill Pavilion gnotobiotic mouse facility, University of Pennsylvania. NOD mice were

colonized with the PedsCom consortium[60] by cohousing germ-free female NOD mice

with PedsCom-colonized C57/BL6 female mouse for 2 weeks. Colonization of cohoused

PedsCom NOD mice was confirmed using qPCR probes for each PedsCom taxa as

previously described[60]. All germ-free and gnotobiotic mice were maintained under strict

germ-free conditions. Mice at Hill Pavillion were housed in flexible film isolators (Class

Biologically Clean, WI, USA), fed autoclaved LabDiet 5021 (Cat# 0006540) ad libitum,

and caged on autoclaved Beta-chip hardwood bedding (Nepco, NY, USA). Sterility

checks were regularly performed on isolators each month and additionally prior to any

transfer of animals. Freshly collected pellets were cultured on brain heart infusion (BHI)

(Oxoid, UK), NB1, and Sabouraud media for 65–70 hours at 37°C under aerobic and

anaerobic conditions with positive and negative control samples. All mice were housed

in American Association for the Accreditation of Laboratory Animal Care (AAALAC)-

accredited facilities at the University of Pittsburgh or at the University of Pennsylvania.

All animal procedures were conducted in accordance with the Guide for the Care and

Use of Laboratory Animals under protocols approved by the Institutional Animal Care

and Use Committees (IACUC) at the University of Pittsburgh and University of

Pennsylvania.

Salmonella infection model

129X1/SvJ mice, aged 8-16 weeks, were orally pretreated with streptomycin 20 mg. The

following day, mice were orally infected with 2.5 x 10^8 colony-forming units (CFU) of

Salmonella enterica serovar Typhimurium (Strain SL1344)[81] diluted in PBS. Mice were

sacrificed at four hours post-infection and four days post-infection, and cecal and colon

contents were collected at both time points.

METHOD DETAILS

Preterm infant stool microbiota extraction

Approximately 20 to 200 mg of stool is weighed and recorded. Fecal material is

homogenized by resuspending with vigorous vortexing and pipetting in 1 mL phosphate

buffered saline (PBS, pH 7.4) then pelleted by centrifugation (10 minutes, 9000g).

Supernatant is collected and stored (-80°C) for testing fecal waters. To remove

undigested milk protein/fat, the fecal pellet is washed twice in 2 mL of 5% Percoll/0.15

M NaCl, pelleted (10 minutes, 10,000g) and resuspended in 1 mL PBS. To separate

eukaryotic and prokaryotic cells, the sample is centrifuged (5 minutes, 50 g), filtered (20

μm cell strainer; Fisher Scientific #NC9912653) and pelleted (10 minutes, 9000g). If the

sample is not immediately to be processed for MicFLY, it is resuspended in 1 mL of

50% glycerol (diluted in molecular biology grade water), aliquoted into replicate tubes

with 200-500 uL each, and frozen (-80°C). Final volume of resuspended microbiota

(1000 uL in 50% glycerol) is recorded for absolute abundance calculations.

Mouse stool and intestinal microbiota extraction

Microbiota extraction for mouse stool is similar to preterm infant stool but simplified

because mouse fecal matter contain less undigested material. Thus, the Percoll

washing steps can be omitted, and stool homogenate can directly proceed to the low-

speed step first (5 minutes, 50 g) before continuing protocol exactly as for preterm stool.

Microbiota extraction from mouse cecal and colonic contents from the *Salmonella*

infection model is exactly as for mouse stool.

MicFLY protocol

MicFLY can be performed in either 96-well plates or microcentrifuge tubes. The protocol

for 96-well plates is for microbiota extracted from stool. The protocol is adapted to

microcentrifuge tubes if extracting bacteria from whole cecal or colonic contents. PBS is

used at pH 7.4 unless otherwise indicated as alkaline PBS, which is pH 8.0.

Viability staining:

Aliquoted extracted microbiota stored at -80°C is removed to thaw. For a single test on

preterm infant microbiota, 200 uL of sample is pipetted to a new microcentrifuge tube

with 1 mL PBS. For a single test on mouse microbiota, 50 uL of sample is transferred. If

several tests are needed (i.e., heat-killed vs. non heat-killed samples or for single color

controls), several tubes are prepared. To calculate absolute abundances, the volume of

each sample is recorded. Samples are pelleted (10 minutes, 10,000g), resuspended in

200 uL PBS and transferred to a 96-well U-bottom polypropylene plate (Greinier Bio-

One #650201). For heat-killed mouse stool experiments, prior to transferring to multiwell

plate, the resuspended sample was heated at 95°C on a dry heat block for 20 minutes

then transferred to plate. Sample is pelleted in the 96-well plate (10 minutes, 4200g)

and resuspended with 100 μL fixable viability dye eFluor780 (1:1000 in PBS; Thermo

Fisher Scientific #65-0865-18) and incubated on ice in the dark for 30 minutes. Sample

is washed with PBS and ready for surface protein staining. If fluorescent antibodies will

not be added, sample proceeds directly to PFA fixation step.

Fluorescent antibody staining and cross-linking

Fluorescent antibodies are added and incubated on ice for 1 hour in the dark. For IgA

binding tests in preterm infants, goat anti-human IgA (alpha chain) polyclonal antibody

CF633 (Biotium #20427) or mouse anti-human IgA monoclonal antibody VioGreen

(Miltenyi clone IS11-8E10) are used at 1:10 dilution. For isotype controls, goat anti-

chicken IgY antibody CF633 (Biotium # 20126) or mouse IgG1 isotype control antibody

VioGreen (Miltenyi clone IS5-21F5; #130-113-207) was used at 1:10 dilution. For

preterm infant IgG binding tests, mouse anti-human IgG monoclonal antibody BUV737

(BD BioSciences clone G18-145) is used at 1:20 dilution. Antibodies are diluted in 10%

normal goat or normal mouse serum in PBS, or both if combining anti-IgA and anti-IgG

antibodies. For mouse IgA binding tests, goat anti-mouse IgA (alpha chain) polyclonal

antibody DyLight488 (Abcam #ab97011) is used at 1:10 dilution with 10% normal goat

serum in PBS. After incubation, sample is washed three times with alkaline PBS (pH

8.0), which is necessary for downstream BS3 crosslinking. After washing, sample is

resuspended with 50 μL of PBS at pH 8.0 and mixed with 50 μL of 2 mM BS3 (diluted in

alkaline PBS; final concentration = 1 mM) to crosslink antibodies to the bacterial cell

surface. Sample is incubated on ice in the dark for 30 minutes. After incubation, BS3 is

washed from sample with PBS.

Bacterial paraformaldehyde fixation

Sample is resuspended in 100 μL of PBS then mixed with 100 μL of 8% PFA (final

concentration 4% PFA). Sample is incubated on ice in the dark for 30 minutes, then

washed with PBS.

Bacterial permeabilization

To permeabilize bacteria prior to adding initiator probes, fixed bacteria are incubated at

37°C for one hour with 100 μL of 0.1 mg/mL lysozyme (Sigma Aldrich #L6876-1G) and

0.01 mg/mL lysostaphin (Sigma Aldrich #L7386-15MG) diluted in 10 mM Tris-HCl pH

7.0. For mRNA detection, 0.5 units/μL NxGen RNAse inhibitor (BioSearch Technologies

#30281-1) is also added to permeabilization buffer, and proper precautions are taken

using RNAse-free reagents or DEPC-treated water to dilute buffers going forward. After

lysozyme/lysostaphin incubation, 100 uL of PBS with 0.1 % Tween (PBS-T) is added.

Sample is pelleted (10 minutes, 4200g), then washed again with PBS-T.

Hybridization Chain Reaction (HCR)

For information on probe design see section "Ribosomal RNA database and rRNA

initiator probe design". Prior to adding initiator probes, sample is resuspended with 100

μL pre-warmed probe hybridization buffer (Molecular Instruments) for a minimum of one

hour (37°C). While sample is pre-hybridizing, the probe solution containing the panel of

initiator probes is prepared. For a single test, 1 µL of initiator probe from 4 µM working

stock solution is used. If competitor probes are needed, then 1 µL of a competitor probe

from a 20 µM working stock solution is used. All initiator probes in the probe panel (1 µL

each probe) are pooled together into 50 µL of probe hybridization buffer per sample.

After the pre-hybridization incubation step, 50 µL of pooled initiator probes in probe

hybridization buffer is added to each sample and incubated overnight (37°C, covered).

For single color controls, *Eubacteria* initiator probes are used as a positive control to

confirm signal detection.

After hybridization, the sample is washed three times with 200µL probe wash buffer

(Molecular Instruments; 10 minutes, 4200g) with 5 minute 37°C incubations between

washes. Sample is resuspended with 25 µL pre-warmed (room temperature)

amplification buffer (Molecular Instruments) to prepare for amplification (30 minutes,

room temperature). During incubation, fluorescent hairpins are prepared. 0.35

µL/sample of each fluorescent hairpin (stored at -20°C as a 12 µM solution in molecular

biology grade water) is snap-cooled in PCR strip tubes in a thermocycler (95°C for 90

seconds, then 22°C for 30 minutes) then pooled into 15 µL amplification buffer per

sample. When pre-amplification is complete, pooled hairpins are added and incubated

for 4-18 hours (room temperature; covered)

After hairpin amplification, samples are washed with 200 µL 5X SSCT (saline sodium

citrate with 0.1% Tween) three times, (10 minutes, 4200g) and resuspended in a final

volume of 200 μL. This final volume must be recorded for absolute abundance

calculations.

Spectral flow cytometry

Spectral flow cytometry was performed on the Cytek Aurora (Cytek Biosciences).

Scatter gain settings were: FSC-A = 250, SSC-A = 50, and SSC-B = 50. Threshold

settings used the "AND" function, set to 1500 for FSC-A, 1500 for SSC-A, and between

500-1000 for the peak fluorescence channel corresponding to the *Eubacteria*

probe/hairpin pair used in the spectral panel. For example, when *Eubacteria* probe with

an Alexa Fluor 405-conjugated hairpin was used, a threshold was set on the V2

channel. Stopping gate is placed on at least 20,000 live *Eubacteria*+ events. To analyze

rare events, 100,000 live *Eubacteria*+ events are captured. Some preterm infant stool

had very few bacteria with a minimum of 5000 live *Eubacteria*+ events captured. Cytek

volumetric counting is automatic and obtained using $VOL function on FlowJo v10.10.

Bacterial culture

For single culture experiments using multiplexed probe panels to target all major gut

bacterial taxa, facultative and obligate anaerobic species were grown under a range of

conditions, detailed in **Supplemental Table 4**. All facultative anaerobic bacteria, except

L. gasseri, were streaked from -80°C stocks (25-50% glycerol in LB media) onto LB,

MacConkey, or tryptic soy agar (TSA) plates and incubated at 37°C overnight prior to

growing in liquid culture. A single isolate was then inoculated into 5 mL LB broth and

incubated shaking at 37°C for ~18 hours. *L. gasseri* was directly seeded into 5 mL MRS

broth from frozen stock and incubated shaking at 37°C for 48 hours. For obligate

anaerobic bacteria, cultures were grown in an anaerobic chamber (0 ppm O_2, 2.0% H_2).

Anaerobic bacteria were streaked from –80°C glycerol stocks onto TSA with 5% sheep

blood or modified clostridial agar and incubated at 37°C for 24-72 hours. A single isolate

was used to inoculate 2-5 mL broth (reinforced clostridial medium or peptone yeast

extract broth; Bifidus Selective Medium Broth for *Bifidobacterium* species) and

incubated for another 24-96 hours (**Table S5**). For some strains in original manufacturer

vial, bacteria were either fixed directly or inoculated into broth immediately. For

PedsCom isolates, bacteria were grown as previously described[60]. The volume of

bacteria culture used for MicFLY testing was the amount needed to obtain a visible

pellet which differed depending on species and was sample-specific (~20-200 μL of

overnight culture for facultative anaerobes; ~100-1000 μL for obligate anaerobes). Prior

to MicFLY testing, all cultures are washed with PBS, and fixed with 4% PFA.

For mixed culture experiments, bacteria were grown separately and combined after

fixation. For *E. coli*, *S. aureus*, and *B. breve* mixed culture experiments, 25 μL from

overnight *E. coli* and *S. aureus* cultures were washed, fixed, and combined with 250 μL

of fixed *B. breve* from a 1 mL culture. For *E. coli* and *Stm* mixed culture experiments, 4

x 10^7 CFU of each bacteria were used.

Reporter GFP *E. coli* and mCherry Stm cultures

For GFP mRNA detection experiments, DH5α *E. coli* strain with arabinose-inducible

GFP carried on p006-GFP-pBAD plasmid with a kanamycin resistance gene (Addgene

#108315) is streaked onto kanamycin agar plates (50 µg/mL) and incubated overnight

at 37°C. An isolate is inoculated into 5 mL kanamycin LB broth (100 µg/mL) and

incubated shaking overnight at 37°C. A 1:100 dilution of overnight culture is seeded into

fresh kanamycin LB broth the next day, and bacteria are grown to log phase (3-4 hours

at 37°C) prior to arabinose induction. For dose-response experiments, different *E. coli*

cultures were induced for three hours with arabinose final concentrations of 0%,

0.0001%, 0.0005%, 0.001%, 0.005%, 0.01%, 0.05%, or 0.1% (w/v). As a control, non-

transformed *E. coli* (605 strain[82]) is grown and tested in parallel in kanamycin-free

media. For time-course experiments, two *E. coli* cultures were grown and induced with

either 0.01% or 0% arabinose. A fraction of each culture was assayed at defined

intervals post-induction (0, 15, 30, 45, 60, 120, 180, and 240 min). In induction

experiments with spent media, three cultures of GFP *E. coli* were induced with 0.01%

arabinose for 1 hour, 4 hour, or 18 hours. At each time point, bacteria were pelleted (5

minutes, 9000g), and 'spent' media was collected, filtered through a 0.2 micron syringe

filter and stored at 4°C. Three new GFP *E. coli* isolates were grown, and overnight

bacteria were used to seed a fresh culture grown to log phase. Spent media from the

different time points were used to induce *E. coli* for one hour.

For mCherry mRNA detection, *Stm* (wildtype or mCherry-expressing strain) is streaked

onto MacConkey plates and incubated overnight at 37°C. An isolate is inoculated into 5

mL LB broth and incubated shaking overnight at 37°C. 250 µL of overnight culture is

seeded into 5 mL of fresh LB broth the next day, and bacteria are grown to log phase

(3-4 hours at 37°C) prior to assaying transcripts.

100 µL of log phase culture is used for MicFLY testing in these experiments. To prevent

mRNA degradation, isolated bacteria are immediately fixed (4% PFA) and tested.

GFP sorting and quantification

To quantify the 16S or ompX gene from GFP-expressing *E. coli*, *E. coli* were induced

with 0.01% arabinose for one hour as described above. For sorting, 200 uL of induced

bacteria are washed and 4% PFA-fixed, then undergo the MicFLY protocol using a

panel with *Eub338* probe/B3-Alexa Fluor546, *Enterobacteriaceae* probe/B2 Alexa-Fluor

546, and GFP mRNA probe/B1-Alexa Fluor 647. MicFLY-treated bacteria are

resuspended in 500 uL of 5X SSCT in FACS tubes and sorted on the Cytek Aurora Cell

Sorter (threshold on FSC-A = 500). 2,000,000 events, or as many as possible until

sample ran out, were sorted separately for Eub338+GFP+ and Eub338-GFP-

populations, and bacteria are pelleted (5 minutes, 9000g). To extract DNA from sorted

cells, nucleic acids are first decrosslinked by resuspending pellet with lysis buffer

(50 mM Tris pH 8.0, 200 mM NaCl, 25 mM EDTA) and proteinase K (0.4 ug/uL;

BioSearch technologies #MPRK092) then incubated at 60°C for 1 hr. QIAamp

PowerFecal Pro DNA Kit (Qiagen) was then used according to manufacturer's

instructions, with a minor modification in the initial lysis step: 500 µL of solution CD1

was added to each sample, vortexed for 15 seconds, and centrifuged (1 minute, 5000g)

before proceeding with the standard protocol. Extracted DNA is stored at -20°C.

To quantify bacterial genes by qPCR, input DNA was normalized by using a volume

corresponding to 200,000 sorted events. SsoAdvanced Universal SYBR Green

Supermix (BioRad #1725271) was used according to manufacturer's instructions.

Briefly, 5 uL SYBR green, 1 uL forward primer (20 uM), and 1 uL reverse primer (20 uM)

were mixed with diluted DNA in nuclease-free H2O up to a 10 uL reaction. Forward

primer for ompX gene was 5'-GCTTTCACCGCAGGTACTTC-3'. Reverse primer for

ompX gene was 5'-ATGCTTGCCCAGTCGTTAAT-3'. Forward primer for 16S gene was

5'-GTGCCAGCMGCCGCCGTAA-3'. Reverse primer for 16S gene was 5'-

CCGYCAATTYMTTTRAGTTT-3' Data acquisition was performed on the CFX Duet

Real-Time PCR System (Bio-Rad).

MicFLY vs. Syto BC quantification

To compare MicFLY and Syto BC methods to colony-forming units (CFUs), isolates of

E. coli, *K. pneumoniae*, *S. aureus*, and *E. faecalis* are grown as described. To serially

dilute bacteria, 100 µL of overnight culture is diluted into 900 µL LB broth (dilution factor

10^1), and repeated for each previous dilution until dilution factor 10^{10} is reached. To

count CFUs, 100 µL of each dilution is plated onto MacConkey agar (*E. coli* and *K.*

pneumoniae) or LB agar (*S. aureus* and *E. faecalis*) in duplicate (thus increasing the

dilution factor another tenfold), followed by overnight incubation at 37°C. CFUs are

calculated the next day. The remaining 900 uL of diluted sample is pelleted (5 minutes,

9000g), washed with PBS, fixed with 4% PFA on ice for 30 minutes and resuspended in

900 µL PBS. 100 µL from each dilution tube is set aside at 4°C for Syto BC testing. A

100 µL is aliquot is then assayed with MicFLY. The other fraction is then incubated with

Syto BC (Thermo Fisher #S34855) at 1:400 dilution for 15 minutes on ice, washed 3

times with PBS, and resuspended in 100 μL PBS. Samples are run in parallel according

to our MicFLY spectral cytometry settings. Counts from colony growth are then

compared to results from the flow cytometer.

Bacterial imaging

To image bacterial cultures, 20 μL of an overnight *E. coli* culture and 200 μL of an

overnight *S. aureus* culture are fixed, mixed together, and undergo HCR according to

the MicFLY protocol using a panel with *Enterobacteriaceae* probe/B2-Alexa Fluor 594

and *Bacilli* probe/B4-Alexa Fluor 488. After MicFLY treatment, bacteria are pelleted (5

minutes, 9000g) and resuspended in 5 μL of PBS. The 5 μL bacterial suspensions are

then mixed with 20 μL of 2% low melting point agarose (Thermo Fisher #16520050),

and 10 μL is loaded onto individual wells of a 10-chamber imaging spacer (GraceBio

#470352) on a glass microscope slide. Bacteria are imaged on a Leica TCS SP8

confocal microscope using 60x objective.

***In vitro* breast milk IgA experiment**

In vitro IgA binding to bacteria was performed according to our previous protocol[82].

Briefly, 30 μL of an overnight *E. coli* culture (strain #605) was incubated with or without

IgA purified from breast milk in 10% normal goat serum on ice for 1 hour. After

incubation, bacteria was washed 3 times in PBS then stained with secondary antibody

goat anti-human IgA (alpha chain) polyclonal antibody CF633 (Biotium #20427) in 10%

normal goat serum in PBS on ice for 1 hour. Bacteria were washed 3 times with alkaline

PBS (pH 8.0) to prepare for antibody crosslinking/fixation exactly according to the 1 mM

BS3 and 4% PFA steps of the MicFLY protocol. After PFA fixation, sample was

fractionated into two tubes, in which one proceeds through the rest of the MicFLY

protocol with probe hybridization and HCR, while the other is stored at 4°C. Prior to

running on the spectral flow cytometer, both fractions are stained with Syto BC (Thermo

Fisher #S34855) at 1:400 dilution for 15 minutes on ice then washed 3 times with PBS.

Samples are run according to our MicFLY spectral cytometry settings.

Fecal waters IgA ELISA

Fecal waters were prepared using 1 mL of preterm infant stool homogenate in PBS as

described (see '**Preterm infant stool microbiota extraction**'). Human IgA was

quantified using a commercial ELISA kit (Abcam #ab137980) according to

manufacturer's protocol. Because fecal IgA concentration is unknown, fecal waters were

tested at 1:20 or 1:50 dilutions. Samples for each dilution were assayed in duplicate

wells and averaged. OD was read at 450 nm on a microplate reader (BioTek Synergy

H1). To normalize data, IgA concentration (ng) in 1 mL fecal waters was divided by

original stool mass and displayed as ng IgA/mg stool.

DNA Extraction for PedsCom qPCR and 16S-seq

A stool pellet from each PedsCom mouse was collected into a microcentrifuge tube and

weighed prior to DNA extraction. DNA was isolated using QIAamp PowerFecal Pro DNA

Kit (Qiagen #51804) according to manufacturer's instructions with the following

modification: DNA was eluted in 50 µL of Solution C6 after heating column at 56°C for

~10 min to increase yield. DNA was also extracted from germ-free mouse stool as a

negative control. Eluted DNA was stored at -20°C. qPCR and 16S sequencing were

tested using the same DNA sample.

qPCR absolute quantification for PedsCom mice

TaqMan custom primer and probe sets were designed to target the DNA-directed RNA

polymerase subunit beta (rpoB) gene for each PedsCom member as previously

described[60]. Primer and probe sets were generated using the PrimerQuest Tool

(Integrated DNA Technologies, IA, USA) and compared with the Multiple Primer

Analyzer tool (Thermo Fisher). Absolute quantification of each PedsCom member was

determined by rpoB gene copies per gram feces. Data acquisition was performed on the

CFX Opus 96 PCR system using TaqMan Multiplex master mix (Thermo Fisher). Data

analysis was performed on CFX Maestro ver. 2.3 (Bio-Rad).

16S rRNA gene amplicon sequencing and analysis

Extracted DNA from PedsCom or germ-free stool was sent to Microbiome Insights

(Vancouver, BC, Canada) for 16S gene amplicon sequencing (V4 region) using

515F/806R primers with Illumina adapters. Library preparation followed the Illumina 16S

Metagenomic Sequencing Library Preparation protocol, and paired-end sequencing (2 ×

250 bp) was performed on an Illumina MiSeq instrument. Raw demultiplexed FASTQ

files were returned for downstream analysis.

Sequencing analyses were conducted in QIIME 2 (version 2024.5) with default

parameters unless specified. Demultiplexed paired-end reads were imported via a

PairedEndFastqManifestPhred33V2 manifest file, and quality profiles were inspected for

trimming/truncation parameters. Only forward reads were retained for denoising

because reverse reads exhibited reduced quality and insufficient overlap for merging.

The first five bases of each forward read were trimmed with truncation at 158 bp to

remove low-quality tails. DADA2 denoising applied quality filtering, dereplication and

consensus-based chimera removal, producing an amplicon sequence variant (ASV)

table and representative sequences. Taxonomic assignment was performed with the

feature-classifier plugin (classify-sklearn) using a pre-trained naive Bayes classifier

trained on the 515F/806R V4 region of the SILVA 138 (SSURef NR99) database (QIIME

2 release 2022.10; scikit-learn v1.4.2). Only samples >10,000 reads (post-denoising)

were included with Archaea features removed. ASV counts were normalized to total

reads per sample to calculate relative abundance, which was visualized with the QIIME

2 taxa barplot tool. Relative abundance outputs were exported and plotted in GraphPad

Prism 10.

Ribosomal RNA database and rRNA initiator probe design

A reference database of bacterial rRNA sequences was created using CLC Main

Workbench v8.1 (Qiagen). For 16S rRNA, all sequences were downloaded as a single

FASTA file from BioProject 33175 (Accession: PRJNA33175) from the NCBI RefSeq

Targeted Loci Project, which was imported into CLC Main Workbench. For 23S rRNA,

BioProject 188943 (Accession: PRJNA188943) was used. At the time of our database

generation, 26020 16S sequences and 1350 23S sequences were available for

download. Sequence lists were created on CLC Main Workbench to organize 16S

genes manually into folders for each taxonomic rank of bacteria relevant to our study,

classified based on the List of Prokaryotic names with Standing in Nomenclature

(LPSN) which complies with The International Code of Nomenclature of Prokaryotes

(ICNP). These organized 16S files are available for download in the **Data S1**. Though

not all bacteria were organized into taxonomic categories in our database, we included

the ones most relevant to our studies and the human gut microbiota.

To design rRNA initiator probes, CLC is first used to perform gene alignments on 16S or

23S rRNA for target taxa along with a number of closely related "off-target" bacteria.

Several 16-24 nucleotide long regions of interest that perfectly match target taxa, but

not off-target taxa, are selected as 'candidate probes' to cross-reference against the

rRNA database. The "Find binding sites and create fragments" tool is used, the

corresponding rRNA database is selected, and each candidate probe is tested

separately, limiting maximum mismatches displayed to 2. To select a probe for MicFLY

testing, the probe must have 0 mismatches to target bacteria only. Probes with 0

mismatches to off-target bacteria were discarded for further evaluation. Additionally,

there must be a limited number of off-target bacteria with 1 mismatch to the candidate

probe, such that competitor probes can feasibly be generated to block all possible

mismatches on off-target bacteria. Competitor probes were generated by creating

sequences that have 0 mismatches to off-target bacteria and pooled with initiator

probes during the hybridization step at 5x concentration (20 µM working stock

concentration for competitor probes; 4 μM working stock concentration for initiator

probes). From empirical observations, 2 mismatches to off-target bacteria do not result

in detectable signal from probe hybridization. If a candidate probe has mismatches with

several target bacteria, additional probes can be designed to expand coverage at that

site, thus making a "probe set" (**Table S1**). Once a candidate probe(s) meets selection

criteria, its reverse complement is generated to enable binding to the positive-strand

16S rRNA. To complete the initiator probe design, an initiator sequence must then be

tagged to the 3' end of the reverse-complement probe, including to any other probes in

the same set.

To design additional pan-bacterial 16S rRNA initiator probes (Eub841 and Eub1491),

gene alignments were performed on a few bacteria from diverse phyla. Sites that were

the most conserved were selected as candidate probes. Eub841 (paired with a second

probe for expanded coverage) and Eub1491 were selected because they bind the same

or greater number of bacterial taxa as Eub338 (>24729 sequences/26020 in our

database; see **Data S2**). Together, these three probes target three different sites along

the 16S rRNA (Eub338 between V2 and V3; Eub841 between V4 and V5; Eub1491

between V8 and V9) and cover 25954/26020 sequences in our database. The 66

bacteria not covered by the *Eubacteria* combined probe set are not found in the

mammalian microbiota (**Data S2**). Cross-referencing all *Eubacteria* probes against

human (Gene ID: 100008588) and mouse (Gene ID: 19791) 18S rRNA, as well as their

mitochondrial 12S (human gene ID: 4549; mouse gene ID: 17724) or 16S rRNA (human

gene ID: 4550; mouse gene ID: 17725), showed that the highest similarity was to the

12S mitochondrial rRNA with a minimum of three mismatches.

Sequences for all rRNA probes in this study are detailed in **Table S1**, and files

displaying binding sites for each probe, including mismatches, are uploaded (**Data S2**).

All probes within a probe set have the same initiator sequence and are pooled to

maximize signal for each target bacteria. The non-split initiator design was used for

probes targeting rRNA[38,39] (**Figure S1**), and these sequences are detailed in **Table S2**.

For mRNA probes, a split initiator design was used (**Figure S1b**), which was created

through the Özpolat Lab in situ HCR probe generator (rwnull/insitu_probe_generator on

Github)[70] for GFP, mCherry, and *Stm* ompA sequences. To compare ompA nucleotide

identity between *Stm* (accession number FQ312003.1/locus tag SL1344_1010) and *E.*

coli (accession number BA000007.3/locus tag ECs_1041), genes were aligned using

CLC Main Workbench v8.1.

Joint-Compositional Tensor Factorization (Joint-CTF)

Joint-CTF is a dimensionality reduction method for longitudinal multi-modal data based

on tensor decomposition. It extends Compositional Tensor Factorization (CTF)[104] and

TEMPoral TEnsor Decomposition (TEMPTED)[126], which are applied to a single data

modality sampled over time. Joint-CTF simultaneously decomposes multiple

longitudinal data modalities measured on the same set of subjects, while

accommodating irregular sampling across subjects. For the analysis presented here,

Joint-CTF was applied to two modalities: (1) absolute abundances of

Enterobacteriaceae taxa and (2) antibody-bound taxa. Each data modality was log-

transformed and z-scored to center the data. The normalized data were then

reformatted into 3D tensors with axes corresponding to subjects, features, and time,

and jointly decomposed. This decomposition generates (a) subject loadings, which

embed each subject into a shared low-dimensional space of *n* principal components

(PCs), capturing cross-modality variation; (b) feature loadings, which are modality-

specific and quantify the relative contribution of individual features to each PC; and (c)

temporal loadings, which are modality-specific and capture the dominant trajectories of

features over time for each PC. To align time points across subjects with irregular

sampling, original time points are projected into a high-dimensional embedding using

kernel methods, creating a common temporal axis for all subjects.

FlowJo analysis

Flow cytometry data were analyzed using FlowJo v10.10.0 (BD Biosciences). FlowJo

UMAP v4.1.1 plugin was used, and consistent parameters were applied across all

datasets (nearest neighbors = 5, minimum distance = 0.1, and components = 2).

UMAPs were generated on concatenated patient files. FlowJo Cluster Explorer was

used to visualize and present all UMAP data.

QUANTIFICATION AND STATISTICAL ANALYSIS

Absolute abundance calculations

Absolute abundance $= \dfrac{(\dfrac{\# \, taxon \, events / V_{Cytek} * V_{HCR}}{V_{input}}) * V_{extracted}}{m_{stool}}$

Where:

• # taxon events = number of events counted for taxon of interest

• V_{Cytek} = total volume (uL) of sample acquired by Cytek

• V_{HCR} = final volume (uL) of sample in 5X SSCT prior to running on flow cytometer

• V_{input} = volume (uL) taken from glycerol-stored microbiota used as input for testing

• $V_{extracted}$ = volume (uL) of glycerol used for final resuspension of extracted microbiota

• m_{stool} = mass (mg) of stool from which microbiota was originally extracted

1003

1004 Example:

1005 $$\frac{\left(\frac{10,000\ Bacilli\ events/10\ uL * 200\ uL}{50\ uL}\right) * 1000}{25\ mg} = 160,000\ Bacilli\ \text{cells/mg stool}$$

1006

1007 If there were 10,000 *Bacilli*+ events in our MicFLY-tested mouse stool sample, the Cytek

1008 volumetric function showed that 10 μL of sample was run through the cytometer, and 25

1009 mg of a mouse stool pellet was originally processed, then according to the protocol, we

1010 would have 160,000 *Bacilli*+ cells/mg stool.

1011

Statistical analysis

All statistical analyses were performed in GraphPad Prism v10.4.1 unless otherwise

indicated. Statistical tests used are in figure legends. Preterm infant group sizes were

determined by the number of available samples within defined criteria in the cohort.

Stool testing and mice experiments were non-blinded. For comparisons between two

groups, unpaired two-tailed Welch's t-tests or Mann-Whitney U tests for nonparametric

data were used. Paired two-tailed t-tests were applied to matched mouse stool samples.

For comparisons among more than two groups, one-way ANOVA with Tukey's multiple

comparisons test was used. Two-way ANOVA with Tukey's multiple comparisons test

was used for mouse infection experiments comparing two time points with two taxa.

Microbiome Analyst[127] generated PCoA plots on beta diversity with Bray-Curtis distance

analysis; pairwise PERMANOVA with Benjamini-Hochberg false discovery rate (FDR)

correction assessed significant differences in distances. Correlation analyses used

Spearman correlation, with r and p-values reported. Lines in scatter plots represent the

best-fit line from simple linear regression analysis. For multiple pairwise tests,

nonparametric Mann-Whitney was adjusted with Holm–Sidak correction. Statistical

significance is defined as $p < 0.05$.

1029

FIGURE LEGENDS

Figure 1 | MicFLY single cell technology identifies diverse, live bacteria with high

taxonomic resolution.

a, Overview of MicFLY workflow (made with BioRender).

b, Representative confocal microscopy images of *E. coli* (magenta; *Enterobacteriaceae*

probe/B2-Alexa Fluor 594) and *S. aureus* (cyan; *Bacilli* probe/B4-Alexa Fluor 488) after

staining by MicFLY protocol.

c, Flow cytometry identification of *in vitro* grown bacteria in a mixed sample (*E. coli*, *S.*

aureus, and *B. breve*) after MicFLY staining. Cells are gated on total bacteria (*Eub338*

probe/B1-Alexa Fluor 647) prior to identification of individual taxa. Experimental design

graphic made with BioRender.

d, Discrimination of pure cultures of *K. pneumoniae* and *K. oxytoca* using species-

specific probes by MicFLY. Cells are gated on total bacteria (*Eub338* probe/B1-Alexa

Fluor 647).

e, Multiplexed detection of 20 *in vitro* cultured bacterial species from diverse phyla

representing the human gut microbiome by MicFLY staining. Each column represents

signal from a unique probe across different taxa. All probes were tested in the same

panel and stained on individual taxa. Cells are gated on total bacteria (as in **c**).

f, Validation of viability dye on pure culture bacteria analyzed with MicFLY. *In vitro*

cultured *Veillonella parvula* bacteria (*Negativicutes* probe/B5-Alexa Fluor 514) are

heated to 95°C for 20 minutes prior to incubation with fixable viability dye eFluor780 and

compared to non-heat killed sample. Flow cytometry plots (left) indicate gating strategy

and histograms (right) indicate staining intensity with viability dye.

g, Validation of viability dye on bacteria from fecal samples analyzed with MicFLY.

Heat-killed and non-heat killed bacteria isolated from specific-pathogen free (SPF)

C57BL/6 mouse stool are compared. Flow plots (left) indicate gating strategy and

histograms (right) indicate viability dye staining to all bacteria (*Eubacteria* combined

probe set/B5-AF514; center right) or *Bacteroidales* (*Bacteroidales* probe set/B17-Alexa

Fluor 405; far right). All flow cytometry analyses show representative plots from multiple

independent experiments. Numbers in plots represent percentage of events inside the

gate. All histograms are normalized to mode.

1061

Figure 2 | MicFLY quantification and viability assessment of a predefined

microbial consortia.

a, MicFLY staining and spectral flow cytometry resolves all taxa within a defined

bacterial community. Fecal samples were isolated from gnotobiotic Eα16/NOD mice (n

= 5; ages 4-20 weeks old) colonized with a nine-member (PedsCom) bacterial

community (described in image; left). Shown are representative flow plots from MicFLY

analysis at the family-level, gated on total bacterial cells using the *Eubacteria* combined

probe set/B17-Alexa Fluor 405.

b, The composition of PedsCom fecal samples was analyzed by MicFLY, quantitative

PCR and 16S rRNA gene amplicon sequencing (16S-seq; V4 region). Stacked bar

charts depict relative abundance calculated by each method. To calculate relative

abundance for qPCR, the number of counts for a taxon was divided by the sum of

counts for all measured taxa. PedsCom relative abundance measured by MicFLY was

calculated the same way. 16S-seq relative abundance data were processed using

QIIME2.

c, Principal coordinates analysis (PCoA; Bray-Curtis using MicrobiomeAnalyst[127])

depicts beta diversity measurements for each microbiota measurement method.

Ellipses represent 95% confidence intervals for each group, and statistical comparisons

made using pairwise PERMANOVA with multi-testing adjustments based on Benjamini-

Hochberg (FDR). $p < 0.001$ (**); ns, not significant ($p \geq 0.05$)

d, Absolute abundances of each bacterial family measured by MicFLY and qPCR from

paired stool samples for five mice. Bacterial counts normalized to stool weight (mg), and

statistical comparisons performed using a paired two-tailed t-test. Dashed line

represents limit of detection for that taxon, calculated as mean absolute abundance in

stool from germ-free mice (n = 4). $p < 0.05$ (*).

e, Microbiota viability as measured by MicFLY. Statistical significance assessed using

one-way ANOVA with Tukey's multiple comparisons test. Bars show mean± SEM. p <

0.05 (*), p < 0.01 (**), p < 0.0001 (****). Group with four asterisks is significantly different

from every other group.

DNA extracted from one stool pellet for each mouse (five mice total) was used for both

qPCR and 16S-seq. A second stool pellet from each mouse was tested by MicFLY. For

MicFLY and qPCR, each pellet was tested twice in two independent runs, and data

reflect the average relative/absolute abundance **(b-d)** or average viability **(e)** of both

runs. For 16S-seq, data reflect relative abundances from a single sequencing

experiment **(b, c).**

1097

Figure 3 | MicFLY enables single-cell quantification of bacterial mRNA expression

***in vitro* and *in vivo*.**

a, Plasmid map of pBAD promoter-driven arabinose-inducible GFP mRNA with

kanamycin resistant gene.

b, GFP mRNA and protein levels in *E. coli* induced with increasing concentrations of

arabinose (% w/v), measured with MicFLY. mRNA-targeted probes were used to detect

GFP transcripts, while GFP reporter protein was directly measurable by flow cytometry.

c, Dynamics of GFP mRNA expression over time in *E. coli* following arabinose induction

(+) or removal (−), measured with MicFLY.

d, Flow cytometric detection of mCherry mRNA and mCherry reporter protein in a

Salmonella enterica serovar Typhimurium (*Stm*) strain carrying a genomically-encoded,

constitutively expressed mCherry reporter protein, measured with MicFLY. *Stm* strain

lacking mCherry was used as a control. mCherry transcripts were detected by MicFLY

mRNA-targeted probes, while mCherry reporter protein was directly measurable by flow

cytometry.

e, Detection of *Stm* ompA mRNA in a mixed sample of *in vitro* cultured *Stm* and *E. coli.*

f, Experimental design (left; made with BioRender) of *in vivo* ompA mRNA expression

detection. 129X1/SvJ adult mice (n = 5-6 per time-point) were treated with streptomycin,

then orally infected the next day with 2.5 × 10□ CFU *Stm. Stm*-infected mice were

sacrificed at either 4 hours post-infection (4 hpi) or 4 days post-infection (4 dpi) for

MicFLY analysis of cecal and colonic contents. Representative flow plots (right) depict

gating scheme and ompA mRNA expression for *Stm* or *Bacilli*.

g, Microbiota composition in cecum and colon at 4 hpi and 4 dpi as measured by

MicFLY. Graphs depict relative proportions of live *Eubacteria+Stm+* and live

Eubacteria+Bacilli+ populations. Bars show mean ± SEM. Each dot represents an

individual mouse.

h, Graphs depict the median fluorescence intensity (MFI) of ompA mRNA expression in

live *Eubacteria+Stm+* or live *Eubacteria+Bacilli+* populations at 4 hpi and 4 dpi. Bars

show average MFI ± SEM. Each dot represents an individual mouse.

For *in vitro* tests **(b-e)**, bacteria were gated on *Eubacteria*/B17-Alexa Fluor405+

Enterobacteriaceae/B2-Alexa Fluor 594+. Numbers in flow plots represent percentage

of events inside the gate, and all histograms are normalized to mode. For *in vivo*

experiments **(f-h),** two independent sets of mice were tested (set 1: *n=3*; set 2: *n=2-3*).

Set 1 used probes for *Eubacteria, Enterobacteriaceae, Stm, Lactobacillaceae* (family-

level*)*, and ompA. Set 2 used probes for *Eubacteria, Enterobacteriaceae, Stm, Bacilli*

(class-level), and ompA. For statistical analyses, *Lactobacillaceae* and *Bacilli* data were

combined and reported as *Bacilli*. Statistical tests were performed using two-way

ANOVA with Tukey's multiple comparisons test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$

(***), $p < 0.0001$ (****).

1137

Figure 4 | MicFLY reveals differences in IgA binding to microbiota in preterm

infants fed mother's own milk or donor milk.

a, Study design and sample overview. Graphs summarize longitudinally collected stool.

The day-of-life (DOL) on which a sample was collected is shown by an individual dot.

Colors indicate feeding group (pink = MOM, blue = DM), and each row corresponds to

one infant.

b, Gating strategy to identify IgA-bound bacteria with MicFLY. IgA+ gating was defined

using isotype control antibody (goat anti-chicken IgY CF633).

c, Absolute abundances of *Eubacteria*, *Enterobacteriaceae*, and *Bacilli* populations

measured in MOM- and DM-fed infants and calculated by MicFLY. Bars represent

average total counts ± SEM for pooled samples from DOL 10-21 for each infant. Each

dot represents an individual infant. Statistical analyses use unpaired two-tailed

Welch's t-test.

d, MicFLY measurements of percent IgA-bound bacterial taxa compared by feeding

type. Aggregated data of all samples is shown. Box plots show median, interquartile

range, and minimum to maximum values. Each dot represents a single sample.

Statistical analyses use two-tailed unpaired Mann-Whitney test.

e, ELISA measurements on total concentration of free IgA (ng of IgA per mg stool)

compared between feeding type. Aggregated data of all samples is shown. Box plot

shows median, interquartile range, and minimum to maximum values. Each dot

represents a single sample. Statistical analyses use two-tailed unpaired Mann-Whitney

test.

f, Correlation between bacterial abundance (log-transformed) and percent IgA-bound

bacteria within each taxon, measured by MicFLY. Best-fit line from simple linear

regression is displayed; *r* and *p*-value from Spearman correlation. Each dot represents

a single sample, and each color corresponds to an individual patient. Repeated colors

indicate multiple samples per patient.

For all statistical tests: p < 0.05 (*), p < 0.01 (**), p < 0.001 (***), p < 0.0001 (****). All

analyses done on live cells only.

1167

Figure 5 | Species-resolved longitudinal antibody binding dynamics in preterm

infants who develop NEC.

a, Study design and sample overview. Graphs summarize longitudinally collected stool.

The day-of-life (DOL) on which a sample was collected is shown by an individual dot.

Colors indicate patient group (orange = prospective NEC diagnosis, blue = control), and

each row corresponds to one infant.

b, Absolute abundance of *Enterobacteriaceae* and *Bacilli* species measured by MicFLY

using genus/species-specific probes. Aggregated data of all samples is shown. Box

plots show median, interquartile range, and minimum to maximum values. Statistical

comparisons performed using unpaired two-tailed multiple Mann-Whitney tests with

Holm-Sidak correction.

c, Longitudinal correlation of day-of-life (DOL) and total bacterial counts (log-

transformed) comparing NEC and control infants for amounts of *Eubacteria*+,

Enterobacteriaceae+, and *Bacilli*+ bacteria measured by MicFLY. Best-fit line from

simple linear regression is displayed, and shaded area represents 95% confidence

interval; *r* and *p*-value from Spearman correlation. Each dot represents a single sample,

and each color corresponds to an individual patient. Repeated colors indicate multiple

samples per patient.

d, UMAP projections of species-specific *Enterobacteriaceae*+ events as determined by

MicFLY. Data was concatenated from all patients and timepoints. UMAPs depict

species distribution across NEC and control groups. Plots generated using FlowJo's

UMAP plugin (5 nearest neighbors, minimum distance 0.1, 2 components) and

visualized with Cluster Explorer. Each dot represents a single bacterial cell, which is

color-coded according to species.

e, UMAP projections of species-specific *Bacilli*+ events exactly as in panel **d**.

f, Visualization of IgA or IgG binding intensity overlayed onto UMAP from **d**. Each dot

represents a single bacterial cell; color scale reflects anti-IgA or anti-IgG fluorescence

intensity, scaled from minimum to maximum within the dataset. Species-specific

clusters (*E. coli*, *K. aerogenes*, *K. pneumoniae*, *K. oxytoca*, *E. cloacae*) are shown in

separate cluster-level views.

g, IgA or IgG binding intensity overlays for *Bacilli*+ populations, exactly as in panel **f**.

h, Sorted feature loadings along Principal Component 3 (PC3) from Joint-Compositional

Tensor Factorization (Joint-CTF) analysis using longitudinal absolute abundances of

Enterobacteriaceae taxa and their antibody binding. Feature rankings for only the

IgA/IgG-bound taxa are shown. Features are ordered by their PC3 coefficients,

reflecting their relative association with NEC (orange) or control (blue).

i, Longitudinal correlation of DOL and percent antibody-bound *E. coli* comparing NEC

and control patients measured by MicFLY. Antibody binding is split between IgG- and

IgA-bound bacteria, as indicated. Best-fit line from simple linear regression is displayed,

and shaded area represents 95% confidence interval; *r* and *p*-value from Spearman

correlation. Same samples and color coding as in panel **c**.

j, Longitudinal display of absolute abundance for antibody-bound *E. coli* in NEC and

control patients measured by MicFLY. Total numbers are shown for *E. coli* bound by

IgA, IgG, or both. Same samples and color coding as in panel **c**. All analyses done on

live cells only.

1213

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FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

FIGURE 5

Supplemental Figure S1 | Initiator probe design
a, Designing taxon-specific rRNA initiator probes with accompanying competitor probes to block highly similar but off-target rRNA. Competitor probes do not have an initiator sequence.
b, Non-split initiator and split initiator sequence designs. Non-split initiator sequences are used for targeting rRNA, and split initiator sequences are used for targeting mRNA. Images made with BioRender.

Supplemental Figure S2 | MicFLY effectively targets bacterial 16S rRNA

a, Schematic of the 16S rRNA gene depicting the sites targeted by 'pan-*Eubacteria*' probes, made with BioRender.

b, *E. coli* expressing arabinose-inducible Green Fluorescent Protein (GFP), induced by 0.01% arabinose, was analyzed with MicFLY. Flow plot (left) shows the gating strategy. GFP+ *Eubacteria*+ (green) and GFP-*Eubacteria*- (purple) events were sorted into purified populations and assessed by quantitative PCR for the presence of 16s rRNA genes or *E. coli* ompX, relative to a buffer blank. Each dot represents an experimental replicate.

c, Flow cytometry plots depicting the Forward and Side Scatter properties (left) or *Enterobacteriaceae* probe binding properties of GFP+ *Eubacteria*+ and GFP-*Eubacteria*- bacterial populations identified in b.

d, *E. coli* was grown *in vitro* and used to test the three *Eubacteria* probes by MicFLY. The flow plot (left) shows the gating strategy to identify *E. coli* from debris and the histograms (right) depict the binding of the three *Eubacteria* probes to events from the two gates.

e, MicFLY staining of fecal samples from specific pathogen free (SPF) or germ-free mice with the three *Eubacteria* probes. Flow plots (left) and histograms (right) demonstrate the staining of these probes within the two types of samples. Numbers in flow plots represent are percentage of events within that gate. Numbers in histograms represent mean fluorescence intensity. All histograms normalized to mode.

a

$$Cumulative\ distance\ (C_d)\ =\ \sum_{i=1}^{n} \frac{|\log(y_i) - \log(x_i)|}{\sqrt{2}}$$

E. coli
- Syto BC
- MicFly
- y = x (ideal)

$MicFLY(C_d) = 5.0$
$SytoBC\ (C_d) = 7.1$

K. pneumoniae
- Syto BC
- MicFly
- y = x (ideal)

$MicFLY(C_d) = 3.3$
$SytoBC\ (C_d) = 5.7$

E. faecalis
- Syto BC
- MicFly
- y = x (ideal)

$MicFLY(C_d) = 6.6$
$SytoBC\ (C_d) = 9.2$

S. aureus
- Syto BC
- MicFly
- y = x (ideal)

$MicFLY(C_d) = 6.2$
$SytoBC\ (C_d) = 8.3$

b

1000 uL of total resuspended stool extraction

500 uL for Syto staining

500 uL for MicFLY testing

SytoBC DNA+ cells

MicFLY *Eubacteria*+ cells

SSC-A / SytoBC

SSC-A / *Eubacteria* AF405

Compare total counts in 500 uL

c

$r = 0.721$
$p < 0.0001$

MicFLY count log(*Eubacteria*+ cells)
Syto BC count log(DNA+ cells)

d

$r = 0.033$
$p = ns$

MicFLY count log(*Eubacteria*+ cells)
Input stool mass (mg)

Supplemental Figure S3 | MicFLY permits bacterial quantitation.
a, *In vitro* cultured bacterial samples were assessed for cellular abundance by dilution and plating or flow cytometry, performed with either MicFLY or the DNA stain, SytoBC. Graphs compare the cell number calculated by MicFLY (filled circles) and SytoBC (open circles) to growth on plates. The deviation of each technique from plating was calculated with the equation above and is provided to the right of each graph as cumulative distance (C_d) value. Dashed line represents where y values and x values are equal.
b, Schematic for experiments comparing SytoBC and MicFLY enumeration of fecal samples.
c, Correlation of bacterial abundances calculated from SytoBC and MicFLY data extracted from fecal samples from **b.**
d, Correlation of bacterial abundances calculated from MicFLY to the mass of stool used for MicFLY testing. For (**c,d**), best-fit line from simple linear regression is displayed for log-transformed total abundance data, and shaded area represents 95% confidence interval; *r* and *p*-value from Spearman correlation

Supplemental Figure S4 | MicFLY can identify all bacteria in gnotobiotic mice.

a, Multiplexed detection of 9 *in vitro* cultured bacterial species that together comprise 'PedsCom'. Each column represents signal from a unique probe across different taxa. All probes were tested in the same panel and stained on individual taxa. Cells are gated on total bacteria (as in Fig. 1c).

b, Absolute abundances of each bacterial family measured by MicFLY and qPCR from paired stool samples for 5 mice colonized with 'PedsCom-6' (described in image; left). Bacterial counts normalized to stool weight (mg), and statistics performed using a paired two-tailed t-test. Dashed line represents limit of detection, calculated as the mean absolute abundance in stool from germ-free mice (n = 4). p < 0.05 (*).

c, The probes used for enumerating the taxa present in PedsCom were tested against fecal samples from four germ-free C57BL/6 mice. Number of positive events detected are graphed for each probe. Each dot represents a separate germ-free mouse.

d, Flow cytometry plots depicting the gating strategy (left) and percent of each bacterial taxa within PedsCom-9 that is either live or dead, as determined by staining with fixable viability dye eFluor 780.

e, Comparison of the absolute abundances of live bacteria (calculated by MicFLY) and all bacteria (calculated by qPCR) of family-level taxa from five mice colonized with the 9-member PedsCom. Bacterial counts were normalized to stool weight (mg), and statistical comparisons were performed using a paired two-tailed t-test. Dashed line represents limit of detection for that taxon, calculated as mean absolute abundance in stool from germ-free mice (*n = 4*). p < 0.05 (*).

Supplemental Figure S5 | MicFLY can be used to identify mRNA expression levels in bacteria.
a-e, A plasmid containing an arabinose-inducible Green Fluorescent Protein (GFP) molecule transformed into *E. coli.*
a, Flow cytometry plots depicting GFP and GFP mRNA expression in *E. coli* induced with arabinose (0.01%; bottom row) or without arabinose as control (top row). Histograms (right) show the same data but allow comparison of relative expression over time. Bacteria are gated on *Eubacteria*/B17-AF405+ *Enterobacteriaceae*/B2-AF594+ cells.
b, Graphs depict quantitation of **a.**
c, Media was extracted from the 1-hour (60 minutes), 4-hour (240 minutes) and 18-hour (overnight) timepoints from **(a)** and used to activate GFP expression in new samples of *E. coli*. Shown is expression of GFP reporter protein and GFP mRNA (measured by MicFLY).
d, Flow cytometric plots showing the response of GFP-expressing *E. coli* subpopulations to different concentrations of arabinose at 3 hours post-induction. Shown is expression of GFP reporter protein and GFP mRNA (measured by MicFLY).
e, Flow cytometric plots depicting the effect of a time course of arabinose induction and removal on GFP-expressing *E. coli*. Shown is expression of GFP reporter protein and GFP mRNA (measured by MicFLY).
f, Comparison of the sequence of the ompA gene from *Stm* and *E. coli.* Differences in the sequence of the genes is highlighted in red. Gene alignments made using CLC Main Workbench.

Supplemental Figure S6 | MicFLY retains surface antibody binding on bacteria.

a, A schematic of the experiment is shown on the left (made with BioRender). *In vitro* cultured *E. coli* was incubated with IgA isolated from a breast milk sample, and then crosslinked/fixed with anti-fluorescent IgA antibody (step 1b-2 in Fig. 1a). Bacteria incubated without breast milk IgA, but with secondary fluorescent antibody, was used as a control. IgA binding was quantified before and after MicFLY to assess any loss in binding due to the protocol (step 3 onward in Fig. 1a). Shown is the gating strategy (flow cytometry plots on the left) and level of IgA binding to the bacteria (flow cytometry plots on the right).

b, Fecal samples were isolated from C57BL/6 and IgA-/- mice and analyzed by MicFLY. Flow cytometric plots on the left show the gating strategy. Flow cytometric plots on the right show percentage of IgA binding to various taxa within the mouse microbiota. Center column = C57BL/6; Right column = IgA-/-

c, Quantification of b. Statistical tests were performed using ordinary one-way ANOVA with Tukey's multiple comparisons test. $p < 0.05$ (*).

Supplemental Figure S7 | Gating strategy for combinatorial spectral flow cytometry to identify live antibody-bound bacterial taxa in infant stool.

a, Gating strategy used to identify live bacterial species in stool samples from control or NEC infants using MicFLY. Flow cytometry plots are representative examples from different patient-specific concatenated files, as no single infant contained all taxa shown. Negative gating for all other taxa was applied to each genus/species to ensure it is positive only for its own probe within the *Enterobacteriaceae* or *Bacilli* parent gate. We stacked fluorophores by using combinatorial gating. For example, each *Bacilli* species is negative for *Enterobacteriaceae*/B10-AF660, but positive for *Bacilli*/B2-AF594, and each *Enterobacteriaceae* species is positive for *Enterobacteriaceae*/B10-AF660 but negative for *Bacilli*/B2-AF594. Thus, species-specific probes can be doubled up, as long as they are in different families/orders (e.g., *Staphylococcus/K. aerogenes*/B5-AF514; *Enterococcus/K. pneumoniae*/B4-AF488). Colored boxes highlight strategy used to gate for each taxon.

b, Species-specific IgA and IgG binding patterns across NEC and control infants. Flow cytometry plots were derived from concatenated files of all samples for each individual patient. Shown is IgA/IgG-bound taxa for every patient, based on anti-human IgA (CF633, y-axis) and IgG (BUV737, x-axis). Pseudocolor smoothing with large dot size was applied to improve visualization of low-abundance taxa.

Patients with NEC diagnosis

Bacilli species
- Staphylococcus (yellow)
- Enterococcus (red)
- Streptococcus (cyan)
- Bacilli (other) (dark blue)

Enterobacteriaceae species
- E. coli (pink)
- K. pneumoniae (teal)
- K. aerogenes (light green)
- K. oxytoca (purple)
- E. cloacae (orange)
- Enterobacteriaceae (other) (blue)

Supplemental Figure S8 | Day-to-day UMAP plots of NEC-diagnosed infants.
UMAP plots of composition for both *Bacilli* and *Enterobacteriaceae* species for all stool samples from individual preterm infants who develop necrotizing enterocolitis (NEC). Composite UMAPs (left) represent concatenated data from all available samples per patient, overlaid on live *Eubacteria*/B9-TAMRA+ gated populations. Clusters are color-coded according to taxon, with gray area on the composite UMAP representing unclassified bacteria. UMAPs for each DOL was overlaid on the patient composite UMAP, with gray areas on sample-specific UMAPs corresponding to bacteria not covered on composite. Plots generated using FlowJo's UMAP plugin (5 nearest neighbors, minimum distance 0.1, 2 components) and visualized with Cluster Explorer. Each dot represents a single bacterial cell, color-coded according to species. Antibiotic administration is indicated above each time point where applicable. DOL at which NEC will be diagnosed is marked with a black arrow labeled "NEC dx."

Matched control patients (no NEC diagnosis)

Bacilli species
- Staphylococcus
- Enterococcus
- Streptococcus
- Bacilli (other)

Enterobacteriaceae species
- E. coli
- K. pneumoniae
- K. aerogenes
- K. oxytoca
- E. cloacae
- Enterobacteriaceae (other)

Patient 57 Composite — No abx at birth — DOL 12, DOL 13, DOL 17, DOL 18, DOL 19, DOL 20, DOL 21, DOL 23, DOL 24, DOL 25, DOL 27

Patient 102 Composite — Ampicillin/gentamicin DOL 1-2 — DOL 10, DOL 12, DOL 13, DOL 14, DOL 15, DOL 16, DOL 17, DOL 18, DOL 19, DOL 20

Patient 116 Composite — Ampicillin/gentamicin DOL 1-2 — DOL 15, DOL 16, DOL 24, DOL 28, DOL 29, DOL 31, DOL 34, DOL 35, DOL 36, DOL 37, DOL 38, DOL 39, DOL 40, DOL 41, DOL 42, DOL 45, DOL 47, DOL 48, DOL 51, DOL 52

Patient 133 Composite — No abx at birth — DOL 14, DOL 16, DOL 17

Patient 135 Composite — No abx at birth — DOL 13, DOL 14, DOL 15, DOL 16, DOL 17, DOL 18, DOL 19, DOL 21, DOL 26, DOL 27, DOL 28, DOL 29, DOL 30, DOL 38, DOL 39, DOL 40, DOL 43, DOL 44, DOL 45, DOL 46, DOL 48, DOL 54, DOL 55

Patient 144 Composite — Ampicillin/gentamicin DOL 1-2 — DOL 12, DOL 13, DOL 17, DOL 20

Supplementary Figure 9 | Day-to-day UMAP plots of matched control infants.
UMAP plots are displayed for all stool samples from individual matched control preterm infants.
Parameters/visualization exactly as in Supplementary Figure 8.

Supplemental Figure S10 | Longitudinal absolute abundance of *Enterobacteriaceae* and *Bacilli* species in NEC and control groups.

a, Total counts for *Enterobacteriaceae* species; **b,** total counts for *Bacilli* species. Plots show absolute abundance, measured by MicFLY of each species in stool samples from preterm infants who developed NEC (left) versus matched controls (right). Each panel displays data from a single infant over time post-delivery (x-axis), with DOL of future NEC diagnosis marked by a red dashed line. Antibiotic exposure windows are shaded in gray. Different line colors represent each species. Patients were matched by gestational age (within 2 weeks), sex, delivery mode, and feeding type (mother's own milk [MOM] vs. donor milk [DM]). NEC patients (n=7) and matched controls (n=6) were selected from the prospective longitudinal cohort shown in Figure 5.

Supplemental Figure S11 | Correlation of antibody binding to infant intestinal bacteria to the development of NEC.
a, Graph depicting the percent of IgA-bound *Enterobacteriaceae* and *Bacilli* species in stool samples from NEC (n=7) and control (n=6) infants, as measured by MicFLY. Box plots show median, interquartile range, and minimum to maximum values. Statistical tests were performed using ordinary one-way ANOVA with Tukey's multiple comparisons test. $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), $p < 0.0001$ (****).
b, Percent of IgG-bound *Enterobacteriaceae* and *Bacilli* species as in panel **a**.
c, Graphs of the correlation between bacterial abundance (log-transformed) and percent IgA or IgG bound bacteria within each taxon, measured by MicFLY, for *Enterobacteriaceae* and *Bacilli* in NEC and control groups. Best-fit line from simple linear regression is displayed; *r* and *p*-value from Spearman correlation. Each dot represents a single sample, and each color corresponds to an individual patient. Repeated colors indicate multiple samples per patient.
d-g, Joint-CTF multi-modal dimensionality reduction using three principal components (PCs) derived from absolute abundances of Enterobacteriaceae species and antibody binding over time.
d, Subject loadings, where each point represents an infant's overall multi-modal profile embedded into a shared low-dimensional space. Infants from the NEC (orange) and control (blue) groups are shown across pairwise combinations of the three PCs.
e, Separation of NEC and control groups along each PC from panel **d.** Group differences were assessed using the Mann-Whitney U test; $p < 0.05$ (*).
f, Temporal loadings capturing dominant feature trajectories over time. The x-axis represents a high-dimensional projection of DOL, where original DOL values (1-55) were projected and rescaled to a common 1-100 scale, enabling alignment of irregularly sampled infants.
g, Feature loadings on PC3, reflecting the relative contributions of features to group separation. Based on panel **e,** negative PC3 values (y-axis) associate with NEC, while positive PC3 values with controls.
h, Graph comparing DOL and percent (left) or absolute abundance (right) of antibody-bound *Enterobacteriaceae* in NEC and control patients. Antibody binding is split between IgG- and IgA-bound bacteria, as indicated. Best-fit line from simple linear regression is displayed, and shaded area represents 95% confidence interval; *r* and *p*-value from Spearman correlation. Same samples and color coding as in panel **c**.
i, Longitudinal display of DOL and percent (left) or absolute abundance (right) of antibody-bound *E. cloacae* in NEC and control patients, exactly as in panel **h.**