Published in final edited form as:

J Microsc. 2016 November ; 264(2): 227–237. doi:10.1111/jmi.12438.

Topical injury evaluation of the murine colorectal mucosa using confocal endomicroscopy: a valuable method for assessing mucosal injuries associated with risk of pathogen transmission

Gracie Vargas, PhD^{1,2}, Kathleen Listiak Vincent, MD^{2,3}, Jingna Wei, MD², Nigel Bourne, PhD^{4,5}, and Massoud Motamedi, PhD^{2,6}

¹Department of Neuroscience and Cell Biology, University of Texas Medical Branch, Galveston, TX

²Center for Biomedical Engineering, University of Texas Medical Branch, Galveston, TX

³Department of Obstetrics and Gynecology, University of Texas Medical Branch, Galveston, TX

⁴Department of Pediatrics, University of Texas Medical Branch, Galveston, TX

⁵Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX

⁶Department of Ophthalmology, University of Texas Medical Branch, Galveston, TX

Abstract

Background—Concern regarding the effect of epithelial damage to the colorectal surface and possible impact on sexually transmitted infection transmission prompts the need for methods to evaluate the mucosal microscopic surface in preclinical studies examining such injury. This includes determining the effect of topical HIV prevention products on mucosal barrier integrity. *In vivo* imaging with high resolution endomicroscopy could reveal defects in the mucosal barrier resulting from injury/surface trauma.

Methods—Confocal endomicroscopy was investigated to assess the ability to image surface injury resulting from topical application of a chemical used in lubricants and microbial products. Mice treated with a 50 µl rectal dose of 0.2% benzalkonium chloride (BZK) solution, 1% BZK, or phosphate-buffered saline control for 20 minutes were imaged *in vivo* using confocal endomicroscopy (CE) for assessment of epithelial disruption. Following imaging, mice were sacrificed and rectal tissue evaluated by histology. Confocal images were graded based on degree of disruption to crypt and epithelial microstructure. Histology was graded based on percent of epithelial disruption observed in stained sections. Confocal image features were confirmed by high resolution two-photon microscopy.

Results—Based on quantitative grading of *in vivo* CE images, disruption at the microscopic scale was observed following treatment with BZK, with increased injury occurring with higher dose. Epithelial disruption at the lumen surface, evident between crypts and alteration in crypt structure on the luminal side were observed in CE and confirmed by histology.

Conclusions—High resolution imaging by CE can be used as a noninvasive tool for rapid visual assessment of rectal epithelial integrity following surface injury, potentially providing valuable indication of epithelial injury or trauma.

Keywords

confocal endomicroscopy; colorectal; intravital microscopy; HIV prevention products; mucosal injury; mucosal trauma; two-photon microscopy

Introduction

Defects in the colorectal epithelial barrier could have an impact on transmission of HIV and other sexually transmitted infections, prompting the need for methods to evaluate the microscopic surface in preclinical studies under conditions that could impact the mucosal surface as a barrier. Damage to the epithelium could occur from both physical means as well as chemical means. In fact, topical chemical agents which are designed for use as contraceptive gels, lubricants, and even as topical HIV prevention agents (termed microbicides or non-vaccine biomedical prevention products, nBPs) could potentially impact the mucosal barrier.

HIV/AIDS and other sexually transmitted infections (STIs) are significant global causes of morbidity and mortality. The number of HIV infected people worldwide exceeds 42 million with approximately 48,200–64,500 new infections each year in the United States alone (Prejean *et al.*, 2011, 2008, Hall *et al.*, 2008). Recent findings suggest the rectal mucosa may be particularly vulnerable to HIV infection due to the presence of increased numbers of HIV target cells compared to other parts of the distal gastrointestinal tract; further, rectal target cells have characteristics similar to HIV target cells in the vaginal mucosa (McElrath *et al.*, 2013). The potential impact of enhanced understanding of the rectal mucosal microenvironment and factors associated with injury that could conceivably play a role in HIV transmission is high given anal intercourse is a significant mode of transmission. Currently, the majority of HIV cases are in men who have sex with men (MSM) (Prejean *et al.*, 2011, Hall *et al.*, 2008). There is indication that a significant number of sexually active females (35%) as well as a considerable segment of the teen heterosexual population have potential risk of HIV/STI infection through this route of transmission as well (Mosher *et al.*, 2005, Lescano *et al.*, 2009).

Additional research is needed to better understand the microenvironment of the rectal mucosa as it relates to injury and to the effects of topical products including lubricant gels and prevention products aimed to block transmission of STIs (e.g. microbicides/topical HIV prevention agents). Products designed for topical cervicovaginal use such as contraceptives, lubricant gels, and HIV preventative microbicides, are likely to be applied for rectal use in the absence of rectal-specific products (Gorbach *et al.*, 2012). Chemicals in such products could disrupt the barrier of the mucosa as found in several early products, highlighting the need for studies aimed at evaluating the response of this tissue to such agents. For example, nonoxonyl-9, originally developed as a microbicide and now found in lubricant and contraceptive gel products, leads to extensive exfoliation of the epithelial lining of the

cervicovaginal tract as measured by lavage (Doncel *et al.*, 2004, Cone *et al.*, 2006), biopsy (Doncel *et al.*, 2004, Catalone *et al.*, 2004), and high resolution imaging (Vargas *et al.*, 2009, Vincent *et al.*, 2011). Studies also indicate similar loss of the epithelial lining in the rectum by this product (Phillips *et al.*, 2004, Patton *et al.*, 2002, Phillips *et al.*, 2000). Furthermore, this chemical increases susceptibility to rectal infection by herpes simplex Type II, a co-factor to HIV (Phillips & Zacharopoulos, 1998). Other commercial lubricants used either alone or as carriers for microbicides may also increase vulnerability to STIs (Gorbach *et al.*, 2012, Rebe *et al.*, 2014) with epithelial exfoliation being one observed effect (Fuchs *et al.*, 2007, Sudol & Phillips, 2004). Finally, topical agents could elicit an inflammatory response which recruits cells targeted by HIV and other STIs.

Methods to evaluate rectal mucosal responses to injury are generally limited to white light surface visualization (anoscopy), lavage, and histology (Phillips *et al.*, 2004, Patton *et al.*, 2002, Tabet *et al.*, 1999, Phillips *et al.*, 2000). Each has advantages, such as repeated measures with lavage and disadvantages such as the inability to provide high resolution or subsurface assessment of the mucosal microstructure in real-time. Further, because the mucosal surface response to infection or to perturbing agents can be dynamic and vary with location, methods which provide real-time spatial information and are minimally invasive could provide critical information that static techniques such as histology cannot provide. Confocal endomicroscopy (CE) makes possible *in vivo* imaging mucosal surfaces with subcellular resolution using an endoscopic probe. Contrast for imaging is provided by fluorescent dyes to label tissue targets for the assessment of microstructure, viability, or other cellular indications. Images may be obtained on the order of several frames per second, allowing for real-time and repeated imaging. CE has been applied in a variety of tissues in humans including the gastrointestinal (GI) mucosa (De Palma, 2009, Smith *et al.*, 2008, Hurlstone *et al.*, 2008, Konda *et al.*, 2010, Kiesslich *et al.*, 2007). Murine colonic imaging has been possible through the use of systems having a small distal diameter (<2mm), such as the Cellvizio (Mauna Kau, France) which delivers light through a fiber bundle, as shown in a mouse model for irritable bowel syndrome (Mielke *et al.*, 2015). In the vaginal tract, the method has been applied in preclinical animal studies for assessing microstructure following hormonal or chemical perturbation (Bott *et al.*, 2006, Vargas *et al.*, 2012). While CE has been applied to GI tissues for cancer diagnostics or assessment of cytological abnormalities such as in irritable bowel syndrome, there are no reported studies in rectal tissue for evaluation of the effect of direct injury from external means to the mucosa.

The purpose of this study was to establish the feasibility of using CE for imaging the *in vivo* response of rectal epithelium following rectal application of a known chemical (benzalkonium chloride, BZK) which has been used in 1) contraceptive gels and lubricants (Li *et al.*, 2013), 2) investigated as an antimicrobial agent for topical treatment of vaginal infections (Mosca *et al.*, 2006), and 3) used as a positive control in microbicide assessment studies (Fichorova *et al.*, 2004, Cone *et al.*, 2006, Vargas *et al.*, 2012). It is our goal to examine potential use of CE for *in vivo* quantitative assessment of epithelial injury/disruption with subcellular resolution and which could serve as an *in vivo* preclinical tool for studying effects of injury from external means to the rectal epithelial barrier. The method could be used to study candidate microbicides and other topical agents that may impact the rectal epithelial barrier. In the current study, we establish the feasibility of CE to reveal

changes in mucosal cellular and crypt microstructure and relate results with histology as well as develop a scoring system related to degree of rectal surface injury.

Materials and Methods

Initially, a pilot study was undertaken to generate a training set of images to characterize the microscopic and histologic features of damage induced by topical BZK on rectal tissue. Subsequently, a study in which masked assessment of images and histology was conducted to provide a quantitative measure of damage.

Animal Model

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and conformed to the Guide for the Care and Use of Laboratory Animals. Female Swiss-Webster mice 6–10 weeks of age (Harlan Sprague-Dawley, Houston, TX) were housed in an AAALAC approved vivarium and allowed to acclimate for at least 1 week prior to enrollment in experimental procedures. Animals were housed 5 to a cage with a light/dark cycle of 12/12 with food and water ad libitum and then with fasting overnight for sixteen hours prior to treatment and imaging. Mice were anesthetized with sodium pentobarbital (50 mg/kg delivered by intraperitoneal injection) and placed supine for treatment and imaging. The rectum was gently rinsed with PBS to remove fecal matter. A single application of 25 μ l BZK (0.2% or 1% w/v in PBS) or PBS alone was delivered rectally using a positive displacement pipette (Gilson Microman, Fisher Scientific, Pittsburgh, PA). Twenty minutes later the rectal tract was gently flushed with 1.5 ml PBS using a 3-French (1mm) silicone catheter (SolocathTM, Harvard Apparatus, Holliston, MA) attached to a 3cc syringe. Two 25 μ l applications of 0.05% acriflavine (3,6-Diamino-10-methylacridinium chloride hydrochloride, Sigma) were applied at 5 min intervals, and 5 min after the second application the rectal tract was rinsed with PBS prior to imaging. Acriflavine is known to label nuclei of cells and in the surface gastrointestinal mucosal tissue, cytosol to a lesser degree (Polglase *et al.*, 2005). For the pilot study, mice were treated with 0.2% BZK (n=3) or PBS (n=2). In the subsequent study in which treatments were masked for analysis, animals were treated with 0.2% BZK (n=6), 1% BZK (n=6), or PBS (n=8).

In vivo imaging

A CellVizio confocal endomicroscope (Research model, Mauna Kea Technologies, France) described in the literature (Mielke et al. 2015) was used for the intravital acquisition of images of the murine rectal mucosal surface. The system provides light excitation of the tissue surface with 488 nm laser light via a fiber bundle, with fluorescence emission collected in the spectral range of 505–700 nm. The imaging probe used was 1.5 mm in diameter and provided a field of view of 600 $\times$ 500 μ m, with lateral resolution of 3.3 μ m and axial resolution of 15 μ m, and with *en face* images obtained at the tissue surface. The imaging probe was inserted into the rectum and placed directly on the mucosal surface while acquiring images from twelve separate sites - three sites each on the right, left, anterior, and posterior walls of the rectum. Images were acquired at a frame rate of 12 frames/second and individual representative frames were extracted in TIFF format. Following imaging, mice

were sacrificed and the rectal tissue gently removed and fixed in 10% buffered formalin for 24 hours. Samples were embedded in paraffin and sectioned transversely across the rectal lumen. Serial sections were stained with hematoxylin-eosin (H&E).

Additional imaging using two-photon microscopy

To aid in the interpretation of CE images, *ex vivo* fresh samples of PBS and BZK treated rectum were obtained and imaged by two-photon microscopy using a custom-modified Zeiss LSM 410 two-photon microscope illuminated by a Ti:sapphire (Spectra Physics) operated at 800 nm (140 fs pulses) (Edward *et al.*, 2012) for fluorescence excitation. Images were obtained at the surface and up to 100 μm in depth to reveal the expected crypt structure in *en face* views at both the surface and specific cuts in depth. A 40×1.2 C-Apo water objective was used, providing a $320 \times 320 \mu\text{m}$ field of view, x-y resolution of ($<0.5 \mu\text{m}$) and axial resolution of $1.5 \mu\text{m}$.

Scoring of Images and Histology

CE training set images were reviewed to visually identify features associated with BZK compared to PBS treatment. CE images were also compared to corresponding histology sections to identify image based features of interest potentially associated with damage. Based on this visual assessment of features, CE images were classified as either being normal (0) or abnormal (1), where *normal* images had a typical crypt structure seen following PBS treatment and *abnormal* represented an altered mucosal structure as described below. Graders were trained to identify features associated with these two classes of images. For quantitative evaluation of BZK-induced damage, the subsequent experimental study images were graded by trained graders masked to the sample treatment. Image sets from individual animals were randomized and coded for de-identification of groups or individual mice. Each individual image was assigned a score of normal (0) or abnormal (1) by the grader, and the twelve scores representing the twelve imaged sites in each animal were summed into a single score. Two values were obtained per animal (from two trained graders) and the mean value determined to arrive at an average cumulative animal score. The mean of the cumulative scores in each treatment group was then taken for comparison between groups. As an added measure, inter-rater grading was determined using Kappa analysis resulting in Kappa > 0.71 , indicating the strength of agreement between graders as 'good' (J., 1977).

Photographs of H&E stained sections obtained on an inverted light microscope (IX71, Olympus) were used to quantify epithelial disruption. A 20×0.75 N.A. air objective (UPlanSApo, Olympus) and color CCD detector (DS-QiMc, Nikon Instruments Inc.) were used with image capture controlled by NIS-Elements BR 3.10 (Nikon Instruments Inc.) software. This involved identifying areas of denuded surface epithelium on H&E images and using a curved line tool on image processing software (Metamorph, Molecular Devices) to measure the lengths comprising denuded epithelium. Specifically, a curved line was used to manually outline the entire visible surface on the H&E section to arrive at a total line length. Then curved line segments were used to outline all surface regions missing the columnar epithelium from the mucosal surface and the sum length of these used to define a denuded epithelium length. A ratio of denuded length to total length was used to derive the percent

epithelial disruption. Five slides per animal were measured and the mean value per treatment group calculated. Statistical analysis for treatment group comparison was by two-tailed single factor ANOVA followed by Tukey's post hoc test for both CE and histology measurements.

Results

Representative CE images and corresponding H&E stained histology from a PBS treated mouse are shown in Figure 1. As seen in H&E, the surface epithelium of the lumen is lined by simple columnar cells with striated borders and occasional goblet cells. The epithelial lining is shaped into crypts (glands) with lamina propria surrounding the crypts (filling the interstices between them). In these sections, crypts are seen both in longitudinal view and cross-section due to the orientation of sectioning. In contrast to transverse H&E sections, CE revealed only the *en face* mucosal structure at the surface. In this view, the surface columnar epithelium is brightly stained by acriflavine and crypts projecting in depth appear as dark oval shapes. The *en face* view provided by CE is consistent with the gland structure found in other gastrointestinal tissues using CE devices and fluorophores (Hurlstone et al., 2008, De Palma, 2009, Kiesslich et al., 2007). Glands appear as dark uniform structures and are lined with epithelial cells, the continuous lining of surface columnar cells present between glands with no notable disruption. Each micrograph in Figure 1 is considered a normal grade 0 due to this characteristic, despite occasional floating debris in the mucus, present at bright white dots which are likely nuclei of shed cells in the mucus).

Treatment with 1% BZK resulted in mucosal disruption observed in histology, with CE micrographs indicating significantly altered features (Figure 2). In histology, the vast majority of the mucosal surface was disrupted with missing surface columnar epithelium and disrupted structure of upper regions extending into crypts. Lamina propria surrounding crypts was also exposed at the mucosal surface. In corresponding CE images (Figure 2b), the majority of imaged fields demonstrated heterogeneity and the disrupted surface seen in histology manifested into crypts having a ring-like appearance with a bright outer boundary surrounding the dark gland projecting in depth (panels 1–5,7–11). Disrupted crypts were isolated with regions of exposed lamina propria creating dark gaps between crypts (asterisks in Fig. 1b) also seen in each of these damaged panels. Thus, the treated mucosal surface lacked the largely continuous surface epithelial layer between crypts as seen in PBS treated samples (Figure 1). While this heterogeneity and presence of ring-like crypt structure was present in a majority of imaged fields, some confocal fields maintained a structure similar to that of the PBS case (e.g. panel 12 in the lower right confocal micrograph of Figure 2 as well as panel 6), consistent with segments of surface mucosa maintaining an intact epithelium. The right one-third side of panel 10 has some normal mucosa, but because it is only partial it is considered abnormal. A damage grade of “1” was assigned to micrographs having the majority of the field filled with disrupted crypts evident at ring-like structures and having discontinuities in the columnar epithelium between glands (e.g Figure 2B, and panels 1–5, 7–11 are representative of a damage score ‘1’).

We next used a lower dose of BZK (0.2%) that we expected to produce focal damage to the epithelium. Representative CE and H&E images from a treated animal are shown in Figure

3. Close observation of histology revealed that the surface columnar epithelium was denuded from one-third to half of the mucosa surface leaving the internal lamina propria between crypts exposed and upper crypt structure damaged. In CE, the continuous epithelial lining between glands as seen in the PBS case is evident in some fields, but otherwise disrupted evident as ring-like crypts as seen in the 1% case (panels 1–2,6,10). These are considered damaged fields (grade '1'). Panel 3 shows damage on the left side and in grading could be considered damage since this covers half the field. Other fields demonstrate some surface debris but otherwise lack ring structures as evidence of disruption (4,9,11,12) and likely be considered normal for grading (0). Panels 3,5,7,8,9 would be graded '0'. We found micrographs in the 0.2% case had an overall lower fluorescent signal that was consistent between animals within the group.

Measures of epithelial response to PBS or BZK are shown for CE and histology in Figure 4. After treatment with BZK, CE demonstrates an increase in number of fields showing a disrupted pattern (presence of ring-like objects and debris) within the sampled rectum. The percent of epithelial disruption measured in histology micrographs also demonstrates a dose response, showing increased surface area disruption with increasing dose of BZK. The dose of 0.2% BZK was found to result in statistically higher disruption than PBS but less than 1% BZK.

As noted above, histology revealed that the surface of the rectal epithelium was denuded following both the 0.2% and 1% BZK treatment, exposing normally protected lamina propria to the lumen and altering morphology of crypt upper areas nearest the lumen. Since much of the denuded epithelium occurred between crypts overlying lamina propria, these regions resulted in loss of acriflavine labeling between crypts in CE, resulting in a ring-like crypt pattern in *en face* CE views (acriflavine provides contrast of the epithelial lining of surface and glands, while the continuous layer on the surface would be missing, upper gland linings remain). As confirmation that gland staining pattern at depths beyond the surface would result in a ring-like structure, we performed depth-resolved microscopy of *ex vivo* rectum (PBS and BZK treated) by two-photon microscopy capturing single plane micrographs at the surface and 50 μm in depth at a location beyond the surface (Figure 5). The micrographs confirm the staining pattern of the acriflavine dye is primarily in the nucleus of cells as expected. Near the luminal surface of the PBS treated mucosa, acriflavine-labeled nuclei were seen forming a continuous layer of epithelium between glands (glands seen as round dark areas in Figure 5a). However, at depths beyond the tissue surface, (50 μm depth shown) glands appear as ring-like shapes, particularly because at these depths a single columnar epithelium lining the glands form a 'ring' when viewed *en face*, and the space between glands is now connective tissue at this depth beyond the columnar surface. In BZK treatment, removal of upper epithelium was observed and the luminal surface displayed injured crypts and exposed lamina propria without the overlying luminal surface columnar sheet (Figure 5b, 1% BZK). In the case of 0.2% BZK, features were similar in damaged areas, with focal areas of remaining surface columnar cells.

Discussion

It is known that a number of lubricant and microbial agents used in vaginal products can cause disruption to epithelial surfaces, potentially increasing susceptibility to infection with HIV and other STIs. Many of these substances are also applied rectally and, as occurs in the vagina, could cause disruption to the epithelium as well as recruitment of inflammatory cells that may increase susceptibility to infection. The microenvironment of this tissue contributes to this risk, with an increased number of potential HIV target cells in the lower colorectal region, reaching a maximum in the rectum (McElrath et al., 2013). Despite this potential vulnerability and the potential for infection to occur through anal intercourse, microbicide research to date has primarily focused on the cervicovaginal tract. In this study, we demonstrate CE is suitable for *in vivo* quantitative preclinical evaluation of topical products on the colorectal epithelium and could be used to support studies aimed at testing the vulnerability of this tissue to transmission and infection. Specifically, we show *in vivo* CE can provide quantitative microscopic imaging of the effects of topical agent on the mouse colorectal mucosal surface that results in focal or widespread damage, depending on dose.

Injury to the colorectal mucosa was evident in CE by the appearance of disrupted microstructure which differed significantly from the regular crypt structure found in PBS treated animals, with acriflavine providing the fluorescent optical contrast of nuclei of the epithelial lining. BZK produced disruption to the epithelial surface of the colorectal mucosa within only 20 minutes of application, demonstrating the potential rapid response of the rectal mucosa to external agents. The most striking feature in CE micrographs following BZK treatment compared to PBS treated rectal mucosa was the loss of the continuous columnar epithelium at the lumen surface found between recessing glands. This continuous surface layer was replaced by ring-like structures representing cross-sections of denuded crypts. Another effect was the appearance of disordered structure representing debris from denuded epithelium on the surface. CE allowed sampling of multiple regions *in vivo*, and it was found that the number of fields affected by BZK treatment increased with increased dose of BZK. The lighter appearance of CE micrographs in 0.2% BZK (Fig. 3) was an observation not fully understood, although in these micrographs more surface debris was noted than in other cases and potentially playing a role in image appearance. A CE scoring system produced a cumulative damage score that reflected this observed dose response. Histology confirmed the presence of the disrupted epithelium seen using CE, and by quantitative measure of percent disrupted epithelium, also demonstrated that the degree of damage was related to dose of BZK. Further, histology following *in vivo* imaging was key for explaining the nature of ring-like structures seen in CE. The explanation that the ring-like structures were due to the visualization of gland cross-sections following removal of the upper surface layers was further supported by depth-resolved two-photon microscopy where micrographs of normal rectum taken in depth beyond the surface resulted in ring-like patterns similar to CE but with higher resolution.

The significance of disrupted epithelium found with even the lower dose of BZK and within minutes is that injury to the epithelium may seriously compromise the tissue's primary function as a protective barrier, thereby making the mucosa more susceptible to infection, at least in the acute phase following injury. Wound healing may similarly introduce a

microenvironment with defects in barrier function. In the mouse vagina, epithelial thinning due to exfoliation was in one study best correlated to susceptibility to Herpes Simplex Virus Type II infection with an increase in several risk factors including the number of macrophages in the vaginal lumen, and the levels of inflammatory cytokines in a lavage (Cone et al., 2006). In the rectum, similar damage may occur however the temporal dynamics of mucosal response could differ between the two tissue types (Fuchs et al., 2007, Begay *et al.*, 2011).

A single timepoint was assessed in this study for the purpose of developing an approach to visualize damage by external means (chemical agent in this case) on the rectal mucosa. Images are provided immediately and *in vivo*, allowing one to determine alterations to the mucosa without dissection/biopsy, which is not possible by histology; and further give an indication of the spatial distribution of damage, not possible with methods that utilize lavage. The method could be further expanded to provide *in vivo* real-time imaging in longitudinal studies to evaluate the spatiotemporal response (e.g. damage and wound healing response) of this mucosal tissue to agents applied to the surface or systemically. While damage in this study occurred within 20 minutes it would be of interest to evaluate the temporal extent of the effects of BZK and other lubricants or topical HIV prevention agent.

The ability to conduct *in vivo* imaging in mice is highly desirable since mice are widely used as a model for safety evaluation of topical products including HIV prevention agents/microbicides. With a wide array of available molecular antibodies/reagents developed for use in mice, it remains a valuable preclinical tool for testing a variety of responses and can be a supporting model to help transition to large animal preclinical studies. In the latter case, potentially allowing preliminary testing of approaches to predict responses and optimizing labeling and imaging parameters, minimizing the number of large animals needed for a study. We believe the CE approach could be extended to larger animal models having anatomy on a scale closer to that of humans (e.g. sheep) (Vincent *et al.*, 2013). Ultimately evaluation of products on the mucosal microenvironment is desirable, and human use of CE could be translated for human use. The majority of published literature on CE is in the area of clinical cancer imaging in the gastrointestinal tract. In humans, the available fluorophores are more restricted than in animal studies; most published human studies involve labeling of the GI tract with fluorescein or acriflavine, the stain used in this study.

In summary, confocal endomicroscopy has been applied to provide *in vivo* high resolution assessment of the mouse rectal epithelial surface and demonstration of evaluation of injury caused by BZK, a known irritant investigated for use in vaginal products. Degree of damage was dependent on dose of BZK and was supported by histological confirmation of mucosa which was stripped of surface columnar epithelium across significant areas, also dependent on dose. The approach presented could be used toward preclinical assessment of injury on the rectal epithelium, including chemical and physical injury, and providing a minimally invasive *in vivo* tool to assess dynamics and spatial distribution of damage. The method could serve as an adjunct to other traditionally used histology and molecular biology tools that are possible only at the endpoint of a study.

Acknowledgments

The authors acknowledge Jinping Yang MD for technical assistance and Heather Lander for assistance with manuscript preparation. This work was supported by NIH/NIAID (R33 AI076062, NO1 HD53407, and R01 AI112015)

References

- HIV prevalence estimates--United States, 2006. *MMWR Morb Mortal Wkly Rep.* 2008; 57:1073–1076. [PubMed: 18830210]
- Begay O, Jean-Pierre N, Abraham CJ, Chudolij A, Seidor S, Rodriguez A, Ford BE, Henderson M, Katz D, Zydowsky T, Robbani M, Fernandez-Romero JA. Identification of personal lubricants that can cause rectal epithelial cell damage and enhance HIV type 1 replication in vitro. *AIDS Res Hum Retroviruses.* 2011; 27:1019–1024. [PubMed: 21309617]
- Bott EM, Young IR, Jenkin G, McLaren WJ. Detection of morphological changes of the ovine cervix in response to sex steroids using a fluorescence confocal endomicroscope. *Am J Obstet Gynecol.* 2006; 194:105–112. [PubMed: 16389018]
- Catalone BJ, Kish-Catalone TM, Budgeon LR, Neely EB, Ferguson M, Krebs FC, Howett MK, Labib M, Rando R, Wigdahl B. Mouse model of cervicovaginal toxicity and inflammation for preclinical evaluation of topical vaginal microbicides. *Antimicrob Agents Chemother.* 2004; 48:1837–1847. [PubMed: 15105142]
- Cone RA, Hoen T, Wong X, Abusuwwa R, Anderson DJ, Moench TR. Vaginal microbicides: detecting toxicities in vivo that paradoxically increase pathogen transmission. *BMC Infect Dis.* 2006; 6:90. [PubMed: 16740164]
- De Palma GD. Confocal laser endomicroscopy in the “in vivo” histological diagnosis of the gastrointestinal tract. *World J Gastroenterol.* 2009; 15:5770–5775. [PubMed: 19998496]
- Doncel GF, Chandra N, Fichorova RN. Preclinical assessment of the proinflammatory potential of microbicide candidates. *Journal of acquired immune deficiency syndromes.* 2004; 37(Suppl 3):S174–180. [PubMed: 16419269]
- Edward K, Qiu S, Resto V, McCammon S, Vargas G. In vivo layer-resolved characterization of oral dysplasia via nonlinear optical micro-spectroscopy. *Biomed Opt Express.* 2012; 3:1579–1593. [PubMed: 22808430]
- Fichorova RN, Bajpai M, Chandra N, Hsiu JG, Spangler M, Ratnam V, Doncel GF. Interleukin (IL)-1, IL-6, and IL-8 predict mucosal toxicity of vaginal microbicide contraceptives. *Biol Reprod.* 2004; 71:761–769. [PubMed: 15128598]
- Fuchs EJ, Lee LA, Torbenson MS, Parsons TL, Bakshi RP, Guidos AM, Wahl RL, Hendrix CW. Hyperosmolar sexual lubricant causes epithelial damage in the distal colon: potential implication for HIV transmission. *The Journal of infectious diseases.* 2007; 195:703–710. [PubMed: 17262713]
- Gorbach PM, Weiss RE, Fuchs E, Jeffries RA, Hezarah M, Brown S, Voskanian A, Robbie E, Anton P, Cranston RD. The slippery slope: lubricant use and rectal sexually transmitted infections: a newly identified risk. *Sex Transm Dis.* 2012; 39:59–64. [PubMed: 22183849]
- Hall HI, Song R, Rhodes P, Prejean J, An Q, Lee LM, Karon J, Brookmeyer R, Kaplan EH, McKenna MT, Janssen RS. Estimation of HIV incidence in the United States. *JAMA.* 2008; 300:520–529. [PubMed: 18677024]
- Hurlstone DP, Tiffin N, Brown SR, Baraza W, Thomson M, Cross SS. In vivo confocal laser scanning chromo-endomicroscopy of colorectal neoplasia: changing the technological paradigm. *Histopathology.* 2008; 52:417–426. [PubMed: 17903203]
- JC. *Statistical Power Analysis for the Behavioral Sciences.* Academic Press; Orlando, FL: 1977.
- Kiesslich R, Goetz M, Angus EM, Hu Q, Guan Y, Potten C, Allen T, Neurath MF, Shroyer NF, Montrose MH, Watson AJ. Identification of epithelial gaps in human small and large intestine by confocal endomicroscopy. *Gastroenterology.* 2007; 133:1769–1778. [PubMed: 18054549]
- Konda VJ, Chennat JS, Hart J, Waxman I. Confocal laser endomicroscopy: potential in the management of Barrett’s esophagus. *Dis Esophagus.* 2010; 23:E21–31. [PubMed: 20626448]

- Lescano CM, Houck CD, Brown LK, Doherty G, DiClemente RJ, Fernandez MI, Pugatch D, Schlenger WE, Silver BJ. Correlates of heterosexual anal intercourse among at-risk adolescents and young adults. *Am J Public Health*. 2009; 99:1131–1136. [PubMed: 19008522]
- Li W, Huang Z, Wu Y, Wang H, Zhou X, Xiao Z, Ding X, Xu J. Effectiveness of an optimized benzalkonium chloride gel as vaginal contraceptive: a randomized controlled trial among Chinese women. *Contraception*. 2013; 87:756–765. [PubMed: 23089047]
- McElrath MJ, Smythe K, Randolph-Habecker J, Melton KR, Goodpaster TA, Hughes SM, Mack M, Sato A, Diaz G, Steinbach G, Novak RM, Curlin ME, Lord JD, Maenza J, Duerr A, Frahm N, Hladik F. Comprehensive assessment of HIV target cells in the distal human gut suggests increasing HIV susceptibility toward the anus. *J Acquir Immune Defic Syndr*. 2013; 63:263–271. [PubMed: 23392465]
- Mielke L, Preaudet A, Belz G, Putoczki T. Confocal laser endomicroscopy to monitor the colonic mucosa of mice. *J Immunol Methods*. 2015; 421:81–88. [PubMed: 25960174]
- Mosca A, Russo F, Miragliotta G. In vitro antimicrobial activity of benzalkonium chloride against clinical isolates of *Streptococcus agalactiae*. *J Antimicrob Chemother*. 2006; 57:566–568. [PubMed: 16396918]
- Mosher WD, Chandra A, Jones J. Sexual behavior and selected health measures: men and women 15–44 years of age, United States, 2002. *Adv Data*. 2005:1–55.
- Patton DL, Cosgrove Sweeney YT, Rabe LK, Hillier SL. Rectal applications of nonoxynol-9 cause tissue disruption in a monkey model. *Sex Transm Dis*. 2002; 29:581–587. [PubMed: 12370525]
- Phillips DM, Sudol KM, Taylor CL, Guichard L, Elsen R, Maguire RA. Lubricants containing N-9 may enhance rectal transmission of HIV and other STIs. *Contraception*. 2004; 70:107–110. [PubMed: 15288213]
- Phillips DM, Taylor CL, Zacharopoulos VR, Maguire RA. Nonoxynol-9 causes rapid exfoliation of sheets of rectal epithelium. *Contraception*. 2000; 62:149–154. [PubMed: 11124363]
- Phillips DM, Zacharopoulos VR. Nonoxynol-9 enhances rectal infection by herpes simplex virus in mice. *Contraception*. 1998; 57:341–348. [PubMed: 9673842]
- Polglase AL, McLaren WJ, Skinner SA, Kiesslich R, Neurath MF, Delaney PM. A fluorescence confocal endomicroscope for in vivo microscopy of the upper- and the lower-GI tract. *Gastrointestinal Endoscopy*. 2005; 62:686–695. [PubMed: 16246680]
- Prejean J, Song R, Hernandez A, Ziebell R, Green T, Walker F, Lin LS, An Q, Mermin J, Lansky A, Hall HI. Estimated HIV incidence in the United States, 2006–2009. *PLoS One*. 2011; 6:e17502. [PubMed: 21826193]
- Rebe KB, De Swardt G, Berman PA, Struthers H, McIntyre JA. Sexual lubricants in South Africa may potentially disrupt mucosal surfaces and increase HIV transmission risk among men who have sex with men. *S Afr Med J*. 2014; 104:49–51.
- Smith LA, Tiffin N, Thomson M, Cross SS, Hurlstone DP. Chromoscopic endomicroscopy: in vivo cellular resolution imaging of the colorectum. *J Gastroenterol Hepatol*. 2008; 23:1009–1023. [PubMed: 18557799]
- Sudol KM, Phillips DM. Relative safety of sexual lubricants for rectal intercourse. *Sex Transm Dis*. 2004; 31:346–349. [PubMed: 15167643]
- Tabet SR, Surawicz C, Horton S, Paradise M, Coletti AS, Gross M, Fleming TR, Buchbinder S, Haggitt RC, Levine H, Kelly CW, Celum CL. Safety and toxicity of nonoxynol-9 gel as a rectal microbicide. *Sex Transm Dis*. 1999; 26:564–571. [PubMed: 10560720]
- Vargas G, Patrikeev I, Wei J, Bell B, Vincent K, Bourne N, Motamedi M. Quantitative assessment of microbicide-induced injury in the ovine vaginal epithelium using confocal microendoscopy. *BMC Infect Dis*. 2012; 12:48. [PubMed: 22375797]
- Vargas G, Shilagard T, Johnston R, Bell B, Stegall RL, Vincent K, Stanberry L, Motamedi M, Bourne N. Use of high-resolution confocal imaging of the vaginal epithelial microstructure to detect microbicide toxicity. *The Journal of infectious diseases*. 2009; 199:1546–1552. [PubMed: 19355817]
- Vincent KL, Stanberry LR, Moench TR, Breitkopf CR, Loza ML, Wei J, Grady J, Paull J, Motamedi M, Rosenthal SL. Optical coherence tomography compared with colposcopy for assessment of

vaginal epithelial damage: a randomized controlled trial. *Obstetrics and gynecology*. 2011; 118:1354–1361. [PubMed: 22105265]

Vincent KL, Vargas G, Bourne N, Turner V, Saada JI, Lee G, Sbrana E, Motamedi M. Image-Based Noninvasive Evaluation of Colorectal Mucosal Injury in Sheep Following Topical Application of Microbicides. *Sexually transmitted diseases*. 2013; 40:854–859. [PubMed: 24113407]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Figure 1.

Representative CE images and histology from PBS treated mouse colorectum. A. H&E stained transverse section colorectum showing typical crypt structure and continuous surface epithelium lining the lumen composed of simple columnar cells. B. Single *in vivo* CE micrograph of acriflavin-stained colorectum showing surface crypt structure in an *en face* view. Closely packed nuclei of epithelial cells are visible between crypts which appear as dark circular areas. This micrograph is typical of a normal epithelium. C. Set of twelve CE images acquired *in vivo* in a single PBS treated case. Each of these micrographs would be considered normal due to the regular uniform crypt structure and continuous surface epithelium lining the lumen between crypts. Free floating cellular debris or in the mucus are denoted by #. Scale bar: 40 μ m.

Figure 2.

Representative CE images and histology from mouse colorectum treated with 1% BZK for twenty minutes. A. Transverse H&E showing widespread epithelial disruption with significant interruption to the surface columnar epithelial lining of the lumen. Areas of the lamina propria are exposed to the lumen without overlying epithelial cells (*asterisk) and in disrupted areas, upper regions of crypts are distorted in shape and bulge outward having a ring-like shape in CE (filled arrow). Areas of petechiae are visible. B. Single *in vivo* CE of acriflavin stained colorectum showing significant microstructural damage to majority of surface. C. Set of twelve CE images acquired *in vivo*. In B and C, Nuclear staining pattern is still seen but form a ring-like appearance with gradient toward a dark center at the crypt core (#). Large gaps between crypts are found in areas corresponding to the exposed lamina propria regions of histology (*). In the numbered panels in C only panels 6 and 12 would be considered normal; with panel 10 having normal tissue only in the right 1/3 of the image. Scale bar: 40 μ m

Figure 3.

Representative CE images and histology from mouse colorectum treated with 0.2% BZK for twenty minutes. A. Transverse H&E showing regions of disruption to epithelial lining (*) and upper part of crypt structure as well as regions with an intact columnar layer. B. Single *in vivo* CE of acriflavin stained colorectum showing heterogeneous damage with areas of irregular superficial epithelial cells but less pronounced ring-like appearance (#) to the crypts than seen with the 1% BZK (Figure 2). Additionally, there is surface debris (^) present in the upper right of the image. C. Set of twelve CE images acquired *in vivo*. Some regions show widespread damage (1,2,6,10) and considered damaged (grade '1', with left side of 3 showing disruption and likely also a 1) while other regions show a relatively smooth field (5,7,8) and considered normal grade '0'. Some surface debris is denoted by '^' in 4,9,11,12 with no evidence of disrupted crypts so would likely be considered a '0' grade. Scale bar: 40 μ m

Figure 4.

Top: Results of confocal scoring showing mean cumulative confocal score per treatment group. The cumulative confocal score is representative of the average number of damaged sites out of twelve imaged sites per animal. Bottom: Histology-based measurement of epithelial disruption. Shown is the percent of epithelium disrupted for each treatment group.

** $p < 0.001$

Figure 5.

Acriflavin labeled mouse rectum imaged *ex vivo* by two-photon microscopy. A. Micrograph of the surface taken using a 10× 0.3 N.A. objective providing a field of view of 1.2 mm, approximately two times that of CE. Crypts appear as dark circular regions having a generally regular distribution and continuous fluorescent surface between crypts. B. High resolution micrograph of the rectal surface showing the acriflavin staining is of nuclei of the surface epithelium lining the lumen. C. Micrograph taken at 50 μm in depth beyond beneath the luminal surface, showing that within the lamina propria layer, the single columnar epithelial cells lining the crypt walls give the crypt cross-sections a ring-like appearance. D. Micrograph near luminal surface following 1% BZK demonstrating crypt structure following injury with a similar pattern to that seen in Figure 2B. Two types of nuclei are seen, those of columnar epithelium lining crypts (highly ordered ring configuration) and from the lamina propria having a random spatial distribution. Images are 320×320 μm. Scale bar: 40 μm