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Enhancing Radiation Therapy Through Cherenkov Light-Activated Phototherapy

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Abstract

Purpose: This work investigates a new approach to enhance radiotherapy through a photo therapeutic agent activated by Cherenkov light produced from the megavoltage photon beam. The process is termed Radiotherapy Enhanced with Cherenkov photo-Activation (RECA). RECA is compatible with various photo-therapeutics, but here we focus on use with psoralen, an ultraviolet activated therapeutic with extensive history of application in superficial and extracorporeal settings. RECA has potential to extend the scope of psoralen treatments beyond superficial to deep seated lesions.

Methods and Materials: In vitro studies in B16 melanoma and 4T1 murine breast cancer cells were performed to investigate the potential of RT plus RECA versus RT alone for increasing cytotoxicity (local control) and increasing surface expression of major histocompatibility complex I (MHC I). The latter represents potential for immune response amplification (increased antigen presentation), which has been observed in other psoralen therapies. Cytotoxicity assays included luminescence and clonogenics. The MHC I assays were performed using flow cytometry. In addition, Cherenkov light intensity measurements were performed to investigate the possibility of increasing the Cherenkov light intensity per unit dose from clinical megavoltage beams, to maximize psoralen activation.

Results: Luminescence assays showed that RECA treatment (2 Gy at 6 MV) increased cytotoxicity by up to 20% and 9.5% for 4T1 and B16 cells, respectively, compared with radiation and psoralen alone (ie, Cherenkov light was blocked). Similarly, flow cytometry revealed median MHC I expression was significantly higher in RECA-treated cells, compared with those receiving radiation and psoralen alone (approximately 450% and 250% at 3 Gy and 6 Gy, respectively, $P < .0001$). Clonogenic assays of B16 cells at doses of 6 Gy and 12 Gy showed decreases in tumor cell viability of 7% ($P = .017$) and 36% ($P = .006$), respectively, when Cherenkov was present.

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Conflict of interest: none.

Conclusion: This work demonstrates for the first time the potential for photo-activation of psoralen directly in situ, from Cherenkov light generated by a clinical megavoltage treatment beam.

Summary

This work demonstrates the basic feasibility of a new approach to enhance radiation therapy utilizing Cherenkov light from clinical megavoltage photon beams to photo-activate light-sensitive drugs (eg, psoralen), a process termed “radiotherapy enhanced by Cherenkov photo-activation” (RECA). In vitro studies in B16 melanoma and 4T1 murine breast cancer cells demonstrate that RECA can increase cytotoxicity and surface major histocompatibility complex I. The former represents the potential for increased local control and the latter potential for increased tumor immunogenicity.

Introduction

Psoralens are biologically inert molecules that are well known for anticancer therapeutic effects when photo-activated by ultraviolet (UV) radiation (1). Photo-activated psoralen has been shown to bind to various cellular components, including DNA (17%), intracellular proteins (57%), and lipids (26%) (2). Immunogenic responses have been observed in patients treated with psoralen, with proposed mechanisms including up-regulation of major histocompatibility complex I (MHC I), up-regulation of immunogenic transcription factors (eg, Nuclear factor kappa-light-chain-enhancer of activated B cells, nuclear factor of activated T-cells, Activator Protein 1), and promotion of T-cell development, maturation, and proliferation (1-8). Recently it was also found that psoralen can deactivate the oncogenic protein ErbB2 in breast cancer (9).

Psoralen therapies have wide historical use (1), but applications have been restricted to superficial or extracorporeal settings because of the difficulty in generating UV light in deep tissue, which is a requirement of psoralen activation. Recently, however, x-ray psoralen activated cancer therapy was proposed as a novel solution to the depth limitation by using kilovoltage (kV) x-ray activation of the psoralen through excitement of intermediary phosphor particles that absorb X rays and re-emit UV light (10). Despite this advance, clinical implementation of x-ray psoralen activated cancer therapy is hampered by 2 challenges: (1) the requirement for phosphor intermediaries within the tumor tissue, and (2) the challenges associated with kV irradiation, including high skin and bone doses. In the work reported here, we present an alternative approach that has potential to solve both limitations. The new approach is called “radiotherapy enhanced with Cherenkov photo-activation” (RECA).

In RECA treatment a clinical megavoltage (MV) radiation beam delivers the normal radiation dose to the tumor while concomitantly emitted Cherenkov light (CL), a byproduct of the radiation beam, simultaneously photo-activates administered psoralen specifically within the treatment zone. Cherenkov light is a broad-spectrum UV-visible light produced when charged particles exceed the phase velocity of light within a dielectric material. In MV radiation treatments CL is produced throughout irradiated tissue, with intensity proportional to the local absorbed dose produced from secondary electrons generated throughout the

beam path (11). Cherenkov light intensity per unit radiation dose increases with photon energy (12), suggesting the potential for optimization by using higher-energy photon beams and filtering out low-energy photons. This is investigated here through experimental measurements.

The Cherenkov effect has found recent application in a number of areas, including molecular imaging (13, 14) and phototherapy (15). Most applications utilize injectable radiopharmaceuticals, specifically ^{18}F -fluorodeoxyglucose, that generate CL in tissue from high-speed charged particles liberated during radioactive decay. This approach has the limitations of low Cherenkov production efficiency (compared with the MV range) and distributing radiopharmaceuticals throughout the body.

In this work we present preliminary in vitro investigations into the basic mechanisms and effects of RECA treatment with psoralen, which has well-matched absorption characteristics for CL. The main significance of RECA is the addition of a novel photo-therapeutic component to standard of care radiation therapy treatments, which may enable increased local tumor control and, importantly, the potential amplification of any systemic immunogenic component (as seen with other psoralen treatments) that may impact overall survival.

Methods and Materials

The in vitro effects of RECA were investigated in 2 murine cancer cell lines (B16 and 4T1) using the experimental method outlined in Figure 1. Cells were shielded from ambient light during transportation to the radiation room and during irradiation to ensure cells are only exposed to CL. Well plates of cultured cells were placed on a 3-cm solid water slab and irradiated from below, such that all wells received the same radiation dose, but only half the wells received CL by virtue of a half-beam light block. The light block stopped CL produced in the solid water from reaching the cells on the blocked half of the plate. Luminescence, flow cytometry, and clonogenic survival assays were performed (detailed below) to assess cellular response. The luminescence assay measures total cell metabolic activity, which serves as a surrogate measure of cell proliferation and viability (16). Flow cell cytometry was used to determine change in MHC I expression on the cell surface, which is an indicator of tumor immunogenicity and thus potentially improved visibility to the immune system (2).

Cell culture and preparation

4T1 breast adenocarcinoma and B16 melanoma cells were thawed from -80°C and plated onto a Corning (Corning, NY) 100-mm culture dish at least 2 days before irradiation. Cells were grown in a 5% CO_2 -maintained incubator in Roswell Park Memorial Institute 1640 medium with 10% fetal bovine serum and L-glutamine from GIBCO (Grand Island, NY) at 37°C . One day before irradiation, cells were transferred onto either 96-well (2000-5000 cells per well) or 6-well (100,000 cells per well) clear-bottom plates. Measurements indicate plates have approximately 90% CL transmittance down to a wavelength of 300 nm. Approximately 2 hours before irradiation, cells undergoing luminescence were exposed to various concentrations of trioxsalen (TMP, a psoralen derivative; Sigma Aldrich [St. Louis, MO], T6137) with lights off as specified in Figure 1A. The choice of psoralen

concentrations in the range 10-100 μM was informed by clinical extracorporeal photopheresis therapy (UVADEX, Therakos Inc., West Chester, PA), which utilizes a concentration of 100 μM (8). Cells were transported to the irradiation room in Styrofoam boxes lined with black aluminum foil to minimize exposure to light. After irradiation, treatment solutions were decanted with lights off within 1 hour and replaced with growth medium (Roswell Park Memorial Institute 10% fetal bovine serum). Cells were left to grow in an incubator for 48 hours (luminescence and cytometry) or 1 to 2 weeks (clonogenics) before analysis.

Cell irradiation technique

All lights in the irradiation room were turned off before irradiation. Plates were removed from the Styrofoam container in the dark and positioned on the solid water as shown in Figure 1A for irradiation. Irradiation doses were delivered in the range 0 to 2 Gy for luminescence (96-well plates), 0 to 6 Gy for flow cytometry (6-well plates), and 6 Gy for clonogenics. These dose ranges were chosen in consideration of statistical powers of the assays (17) and variation in the number of seed cells required for 96-well (luminescence) versus 6-well (flow cytometry and clonogenics). The radiation field size was set to cover the plate with an approximately 2-cm margin. A thin (250- μm) light block was placed under half the plate, preventing CL from reaching cells on that side. To verify the CL production from the solid water and the performance of the light block, images were taken with a low-noise iKon-M 934 camera (Andor Tech. Ltd., Belfast, UK) (Fig. 1B). A line profile through a Cherenkov image of the 96-well plate confirmed that CL was illuminating the unblocked wells but not the blocked. A small amount of CL generated in the plate walls does reach cells in the blocked wells, but this is much less than that in the unblocked wells (Fig. 1B).

In vitro assays and analyses

Luminescence assays were performed using the Cell-Titer-Glo Luminescence Cell Viability Assay (Promega, Madison, WI, G7572), an adenosine triphosphate-induced luminescence imaging assay that quantifies the number of viable and metabolically active cells. This enables high-throughput cytotoxicity studies at multiple psoralen concentrations at once. Forty-eight hours after irradiation, media was suctioned off and replaced with 50 μL of Cell-Titer-Glo solution plus 50 μL of media, and cells were left to react with the solution for approximately 15 minutes. Luminescence was then read out with a plate reader. Six wells ($n=6$) were allocated per condition, and all irradiations were performed in a single session.

Flow cytometric analyses were performed on the BD LSRFortessa Cell Analyzer (BD Biosciences, San Jose, CA) system and analyzed using FlowJo (Tree Star, Ashland, OR, version 10.0.7). Cells were first gated on forward and side scatter (FSC/SSC) to exclude small fragments from analysis. Fluorescence of MHC I labeled with allophycocyanin (APC) was then measured. All samples were analyzed on the same day with equal FSC, SSC, APC detector gain voltages and gating. In preparation for flow cytometry, cells were trypsinized and centrifuged 48 hours after irradiation and then resuspended in Cell Staining Buffer at 100,000 cells per milliliter as per BioLegend staining protocol. Cells were stained with anti-H-2K tagged with APC fluorescent dye, which labels MHC I expression on the cell surface, at 0.25 μg per million cells in 100 μL volume, then incubated for 10 to 15 minutes in ice.

Isotype cells were prepared from unirradiated (0 Gy) controls for auto-fluorescence and nonspecific binding control for the antibody.

Major histocompatibility complex I expression histograms, measured as APC fluorescence intensity, were compiled for each treatment condition. The effect of RECA on overall MHC I expression was investigated through pairs of wells treated with the following conditions: 3 Gy with/without psoralen; 6 Gy with/without psoralen; and 0-Gy controls. One of each pair of wells received CL and the other did not by virtue of the light block. A RECA effect would manifest as a difference between cells exposed to CL versus unexposed only when psoralen is present. The Wilcoxon rank-sum test was performed on the null hypothesis that there is no difference between cell populations either exposed or unexposed to CL, with significance set at $P = .0001$. Two wells were allocated per condition, but the 2 wells were combined into 1 sample before analysis. Total number of analyzed events was approximately 200,000 to 500,000 per well.

In addition to the luminescence and MHC I flow cytometry studies, clonogenic survival assays were also performed on 4T1 cells, all with 100 μ M psoralen (trioxsalen) and 1% dimethyl sulfoxide (Fig. 1A). For 6-Gy and 12-Gy irradiations, 3000 and 5000 cells were plated per well, respectively, 30 minutes before irradiation. Ten plates ($n=10$) were irradiated per dose. After 1 to 2 weeks, resulting colonies were fixed with methanol and then stained with crystal violet. ColCount (Oxford Optronix, Milton Park, UK, version 5) was used to count the number of surviving colonies. Student's t test assuming equal variance was performed to compare colony counts with or without CL. Plating efficiency was approximately 15% at 0 Gy, resulting in approximately 450 colonies per 3000 cells plated after 1 to 2 weeks.

Potential for RECA optimization

Experiments were performed using a Varian TrueBeam linear accelerator (Varian Medical Systems, Palo Alto, CA) to investigate the possibility of optimizing the clinical radiation beam such that more CL is gained for the same radiation dose. The experimental geometry is outlined in Figure 2, where an aquarium ($17.8 \times 17.8 \times 17.8 \text{ cm}^3$) was filled with 0.5 g/L of quinine sulfate in water. The quinine absorbs the UV CL and re-emits isotropically as blue light, thereby removing directional sensitivity (and increasing robustness) of the CL intensity measurement. The measured signal from the optical fiber is the relative CL intensity and corresponds to the cumulative integrated signal of the spectrograph over the wavelength range 350 to 500 nm. This signal is from CL-activated quinine and is proportional to the absolute CL fluence (J/cm^2). The phantom was irradiated with 6-, 10-, and 15-MV beams at 600 MU/min, incident laterally with source-to-surface distance = 94 cm and field size = 10 cm^2 . An ion chamber was placed at a depth of 9 cm to measure ionization current (nA), which is proportional to dose rate. An optical fiber was bundled with the ion chamber, directed vertically down and out of the MV beam path. Cherenkov light read-out was made via optical fiber coupled to a LineSpec charge-coupled device Array Spectrometer (Newport Corporation, Irvine, CA, model 78877) with MS125 Grating (400 lines/mm, 325 nm blaze, model 77416). The spectrometer and ion chamber read-outs were simultaneously performed while the MV beam was delivered. Spectrometer integration time

was set at 800 milliseconds per frame with 10 averages, for 8 seconds total acquisition time. Lead radiation shielding protected the charge-coupled device from scattered MV beam and reduced charge-coupled device noise. The measured spectrum from the water phantom was normalized by ion chamber reading, then integrated from 350 to 500 nm (around quinine sulfate emission peak) to obtain the relative CL intensity per dose. Further experiments were performed investigating the potential for filtering the clinical beam to maximize the amount of CL per gray (ie, the amount of CL produced per unit dose). These experiments involved placing a low atomic number block in the beam path, which preferentially absorbs low-energy photons that deposit dose with less CL production. This approach represents a basic method for optimizing the spectrum to maximize the amount of CL per gray.

Results

In vitro assays and analyses

Figure 3A and B show the luminescence assay for cell viability for both B16 and 4T1 cells with (purple, dotted line) and without (green, solid line) RECA. All cells were irradiated with 2-Gy radiation at 6-MV energy but with varying psoralen concentration as indicated. Lines represent least square fits to data points, with 95% confidence intervals indicated by the shaded regions.

Figure 4 shows the MHC I expression results. All cells, including the controls, were exposed to 100 μ M psoralen, representing the baseline control for comparison. In Figure 4A (top), the MHC I expression profiles are compared directly between the unirradiated control with or without 100 μ M psoralen and cells irradiated with the same 3-Gy treatment field but with half the cells exposed to CL by virtue of the light block (Fig. 1). Figure 4A (bottom) shows the same plots but this time for the higher irradiation dose of 6 Gy. Figure 4B compares the median MHC I of all 5 conditions after background correction by subtraction of the isotype background MHC I signal. Statistically significant differences between the CL/no-CL pairs are indicated with an asterisk (*) and confirm that RECA enhancement of MHC I expression only occurs when psoralen is present.

Figure 5 shows clonogenic cell survival data for 4T1 cells.

CL spectrum and potential for RECA optimization

Figure 6A shows the spectrum of the psoralen absorption band, a psoralen–ultraviolet A (PUVA) light source (1), and the CL spectrum in water, obtained from GEANT4/GAMOS Monte Carlo simulations (18). An excellent match is observed in the overlap between the psoralen absorbance peaks and the CL emission wavelengths. The match is noticeably improved when compared with that of the typical PUVA UV light source. Moreover, Cherenkov emits in the critical region of 300 to 320 nm, where the most psoralen-induced damage occurs.

Figure 6B shows the potential for optimizing the amount of CL per unit dose by changing energy and incorporating filters. Relative CL intensity is estimated from cumulative counts from measured spectrum of fluorescent quinine in the range 350–500 nm. Adding a specialized low-Z filter to a flattening filter free 10-MV beam, such as 10 cm polyurethane,

increased the relative CL intensity per dose compared with the standard beam (from 97,000 to 109,000, 13% increase).

Discussion

Figure 3 shows increased cytotoxicity of RECA in both 4T1 and B16 cell lines as measured by adenosine triphosphate luminescence assay. All other conditions being identical, cells exposed to full RECA treatment (with Cherenkov) showed lower cell viability compared with cells that were not exposed to CL (radiation only). Interestingly, as exposure to psoralen increases (TMP at 0-100 μ M), a maximum differential at approximately 50 μ M is observed, after which the differential decreases. The maximum magnitude of difference is 20% and 9.5% for 4T1 and B16, respectively. At low psoralen concentrations (<10 μ M), cell viability is relatively constant regardless of the presence of CL, indicating that CL alone is not able to induce enhanced cytotoxicity over radiation alone.

The MHC I flow cytometry data in Figure 4A reveals a pronounced shift in the MHC I expression profile in RECA-treated B16 melanoma cells. Of particular interest is the observation that the extra shift induced by CL exposure is of equivalent magnitude to that induced by radiation alone (Fig. 4B). A substantial increase in MHC I expression was observed when CL is present, when compared with cells receiving identical treatment but for which the CL was blocked (approximately 450% and 250% at 3 Gy and 6 Gy, respectively, $P < .0001$). This is of interest because B16 cells have been observed to down-regulate surface MHC I as a mechanism for immune escape (19). Increasing MHC I expression, as shown here from RECA, will increase tumor immunogenicity and thus may improve visibility to the immune system. These data are consistent with other observations that MHC I is elevated in photo-activated psoralen treatments (2). They also indicate increased potential for an immunogenic response as reported with psoralen application in extracorporeal photopheresis (1, 2, 8). Further work is required to determine whether these in vitro indications translate into an in vivo setting.

Clonogenic assays of B16 cells at doses of 6 Gy and 12 Gy (Fig. 5) showed a decrease in clonogenic survival in RECA-treated cells of 7% and 36%, respectively, when compared with identically treated cells for which Cherenkov was blocked. This corroborates the cell viability reduction observed in luminescence studies, though higher radiation doses are used owing to lack of response at lower doses. This could mean RECA is more efficient at inhibiting proliferation than inducing cell death, because luminescence assay is more sensitive to proliferation than clonogenic death. Further studies are needed to delve into the biological underworking of RECA.

Figure 6B demonstrates the possibility to optimize the clinical treatment beam for RECA by increasing the relative CL intensity per unit dose. Introducing a low-Z filter (here a 10-cm block of polyurethane) increased CL by 13% for a 10-MV flattening filter free beam.

The results described above indicate that RECA treatment can increase both cell kill and MHC I expression. This result is interesting because prior calculations based on superficial psoralen treatments suggest that the combined dose (defined as the product of CL intensity

and psoralen concentration) from 2- to 12-Gy MV radiation treatments at 100 μM may be 1-2 orders of magnitude weaker than that used in superficial applications that use photo-activation from a standard UV lamp (18). Monte Carlo simulation studies estimate CL dose of 4-8 $\mu\text{J}/\text{cm}^2$ per 1 Gy delivered within the wavelength range of 250 to 850 nm in tissue (simulated without absorption) from a Varian proprietary phase space file for 6-MV radiation treatment beam (12). When considering this, we first note that RECA is a substantively different approach, whereby multifaceted psoralen photo-damage is incurred in addition to radiation treatment. Furthermore, CL has a much more efficient photo-activation spectrum for psoralen than UV lamps used in superficial treatments, as can be seen from the near identical match between the psoralen activation and CL emission spectra (Fig. 6A). The peak wavelengths for cytotoxic DNA–DNA and DNA–protein cross-linking (specifically RecA, a DNA-repairing protein) are 320 nm and 300 nm, respectively, for HMT psoralen (20, 21). The CL spectrum is approximately 12 times stronger in this range when both spectra are normalized by peak wavelength intensity. This observation is consistent with results from an enhanced psoralen superficial treatment incorporating a narrow-band ultraviolet B lamp (311 nm) (22). These considerations support the conclusion that CL is a more efficient light source for psoralen activation and that, in combination with synergistic effects of psoralen and radiation damage, may contribute to the RECA effects reported here. In addition, the potential for increased radio-sensitization of normal tissues in RECA treatment is believed to be of low concern because the spatial distribution of CL matches the dose distribution, which is typically highly conformal in advanced radiation therapy treatments. Because the penetration of CL in tissue is extremely small (millimeters), we do not expect significant psoralen CL activation outside of the planning target volume.

Conclusions

This work demonstrates that RECA, utilizing CL produced by clinical MV radiation photon beams, can increase the cytotoxicity and potential immunogenicity (increased MHC I expression) over radiation therapy alone. The significance of RECA is that it is compatible with, and builds on, current standard of care radiation therapy treatments to achieve both an increased local and systemic effect. Cherenkov light generated from MV treatment beams is well suited for psoralen activation owing to intensity peaking in the short UV. This work demonstrates the potential for optimization of RECA through modifying the clinical MV photon spectrum. Although these results demonstrate that RECA may incur modest additional cytotoxic and MHC I effects, over and above that of radiation alone, the magnitude and translation of any effect in vivo remain to be determined.

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Fig. 1.

Experimental setup for in vitro investigation of radiation therapy enhanced with Cherenkov photo-activation (RECA). (A) Radiation is delivered from underneath to a transparent cell culture plate placed on 3 cm solid water. A light block covers half of the plate, preventing Cherenkov light generated in the solid water from reaching cells on top of the block.

Psoralen concentrations ranged from 0 to 100 μM as indicated. (B) Photographic images of the Cherenkov light emitted in various conditions corresponding to the irradiations in (A). Cherenkov light intensity profile through a 96-well plate confirms that cells that were not under the light block were exposed to approximately 4 times more Cherenkov light than the blocked half (quantified in the line profile). Abbreviation: CCD = charge-coupled device.

Fig. 2.
Experimental setup to measure the relative Cherenkov light intensity per unit radiation dose.

Fig. 3.

Cell-Titer Glo adenosine triphosphate luminescence assay results at varying concentrations of psoralen (TMP) for (A) 4T1 and (B) B16 cells. All cells were exposed to 2 Gy, with half the cells also exposed to Cherenkov light as illustrated in Figure 1A. A maximum of 20% and 9.5% decrease in viability is noted in presence of Cherenkov light for 4T1 and B16, respectively. Quadratic fits are shown with 95% confidence intervals. RECA = radiation therapy enhanced with Cherenkov photo-activation.

Fig. 4. Flow cytometry for B16 melanoma, demonstrating that radiation therapy enhanced with Cherenkov photoactivation (RECA) causes a substantial increase in major histocompatibility complex I (MHC I) expression over and above that caused by radiation alone. (A) Histograms of MHC I expression. All cells received 100 μ M trioxsalen (a psoralen derivative) in 1% dimethyl sulfoxide (unless otherwise specified). (B) Median MHC I expression above isotype control increases for cells receiving Cherenkov light (CL) (purple) compared with no CL (green) only in the presence of psoralen. Wilcoxon rank-sum comparisons are shown for each CL/no-CL (green-purple) pair. *Statistically significant comparisons ($P < .0001$). (A color version of this figure is available at www.redjournal.org.)

Fig. 5.

B16 clonogenic survival data, all cells receiving 100 μ M psoralen. Cells were irradiated in well plates as per the setup in Figure 1A. One sample was lost during processing for 12 Gy (n=9). This design ensures that all cells in a plate got the same radiation dose, but only half were exposed to the corresponding Cherenkov light. Clonogenic survival with (purple) or without (green) Cherenkov light is shown at corresponding doses (6 Gy and 12 Gy). DMSO = dimethyl sulfoxide; RECA = radiation therapy enhanced with Cherenkov photo-activation; TMP = trioxsalen, a psoralen derivative. (A color version of this figure is available at www.redjournal.org.)

Fig. 6.

(A) Relative psoralen absorbance spectrum of 8-methoxypsoralen at 10 µg/mL compared with Cherenkov emission for a 15-MV clinical photon beam in water (obtained using GEANT4/GAMOS Monte Carlo simulations [17]) and a psoralen-ultraviolet A (PUVA) light source. (B) Relative CL intensity per megavoltage radiation dose physically measured from the setup illustrated in Figure 2. Effects of beam energy and polyurethane (low-Z) filter are demonstrated.