UZ LEUVEN | LABORATORIUMGENEESKUNDE

Critically Appraised Topic

Integration of digital microscopy and flow cytometric analysis of solid elements in urine: The best of both worlds and the gate to total automation

- Drop by drop –

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CLINICAL BOTTOM LINE

Urinalysis, one of the oldest medical techniques, has arrived in the 21st century. With the introduction of automated analytical systems, solutions have been developed to automate the counting of particles in urine. Multiple goals are pursued in this development. To reduce the timeframe in which results can be expected (turnaround time, TAT) and thus avoid treatment in cases in which it is not needed. The time-saving effect also frees resources for additional samples, thus increasing the daily throughput of the laboratory. Further, to free the highly qualified personnel for cases in which their expertise is actually needed instead of occupying them with readily distinguishable negative cases. And to allow the generation of reproducible results with standardized procedures. Despite improvements in standardization, errors occur outside of the analytical phase; pre-analytical conditions in particular are much more of a liability.

Quantitative reading of urine strips using reflectometry and CMOS technology is available. Integration of urine concentration parameters allows for correction for urinary dilution. While dipstick test strip analyzers can apparently offer (similarly) quick results, they detect only a certain subset of diseases and infections. To this end, remarkable technical progress can be based on flow cytometry, along with automated microscopic particle analysis. The combined analysis of scattered light and fluorescence allow the rapid identification and differentiation of particles such as leukocytes, bacteria and even iso- or dysmorphic red blood cells. Besides, it allows a reproducible assessment of particle concentration in urine and consequentially a reliable screening procedure for urinary tract infections and hematuria cases. Along with other more arcane parameters like epithelial cells, spermatozoids, casts, crystals and even atypical cells. The added value and complementarity of a digital microscope for qualitative microscopic reviews on indication becomes clear. The sample throughput of such analytical systems combined with the experience in fluorescence flow cytometry and digital microscopy can contribute greatly to the laboratory workflow.

CLINICAL/DIAGNOSTIC SCENARIO

- *Why this topic?*
    - *Validation and implementation of new urine analyzers*

In light of a technical, economic and medical content replacement/renewal, a dossier for new automatic systems for urine test strip analysis and automatic examination of solid elements in urine was issued. The contract led to the investment of new devices that on the one hand read chemical components in urine in a semi-quantitative way and on the other hand count solid elements in urine. The sediment analysis is currently performed with digital microscopy only.

- *What is the current practice and why is it being questioned now?*

Replacement of the current test strip analyzer and microscope is justified below. The current urinalysis combination dates from 2015. There has been a rift between both the test strip reader manufacturer and distributer, with as a result both companies no longer support each other's devices. In addition, the current equipment is showing wear and tear after 7 years of use. Every year, >100.000 test strip analyses and microscopic urine tests are carried out at UZ Leuven. Because of this we are obliged to invest in new equipment, which is justifiable.

Urine analysis is a basic examination and is therefore present in every laboratory. Technical and innovative value of new analyzers should therefore be examined on renewal. The technology of flow cytometric analysis of whole blood, urine and other body fluids has made great progress in recent years. Accurate counts based on size, as well as other characteristics of particles are possible. Robust equipment and software, which makes operation easy for the medical laboratory technologist (MLT), is evolving. In a later phase, connection to an automatic sample transport system (track) is an important degree of automation. The number of microscopic samples to be reviewed manually (i.e. review rate) can be kept to a minimum.

This critically appraised topic addresses the performance of the traditional, routine chemical and manual microscopic urinalysis. Along with a discussion of various (semi-)automated technologies. The focus ultimately is a comparison between the present automated digital microscopy and future flow cytometric analysis of fixed elements.

QUESTION(S)

1) What is the current practice and why is it being questioned now?
2) What are the pitfalls of traditional urinalysis technology?
3) Will urine flow cytometry turn traditional urinalysis obsolete?

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Foreword

Urinalysis is a simple, expeditious, and elementary part of clinical laboratory testing and can provide valuable clinical information in a reliable, safe and cost-effective manner. Physical and chemical analysis of urine and microscopic examination of sediment have proven their usefulness. Urinalysis is indicated for numerous reasons, including in diagnosis of disease, in evaluating the effectiveness of therapy of chronic diseases, and in screening for asymptomatic conditions, congenital or hereditary diseases. The term "urinalysis" includes some or all of the following: macroscopic evaluation (e.g. color, clarity), physical measurements (e.g. volume for timed collections, specific gravity [SG]), chemical reagent strip or tablet testing, and microscopic examination (1).

"Much progress has been made since ancient times,
when urine was poured on the ground and the attraction of insects to it indicated an abnormal specimen." (1)

Pre-analytical conditions (i.e. specimen collection, container, transport and storage) are equally as critical as the technical performance of urinalysis. To ensure specimen freshness urinalysis should be performed within two hours of collection. In case of delay refrigeration is adequate for some chemical components (except photosensitive bilirubin and urobilinogen). Although this can lead to precipitation of amorphous urates or phosphates, which obscure the microscopic field (1). Random specimens may be used for chemical analysis using test strips, the preferred urine specimen (particularly for microscopy) is a well-mixed, first morning, uncentrifuged, 15°C tot 25°C specimen (2,3). This concentrated urine maximizes recovery of sediment elements. It is generally accepted that after standing two hours at room temperature, the chemical composition of urine changes, and formed elements begin to deteriorate. Bacteria can alter glucose concentration, and pH changes can occur if the urine is allowed to stand. Casts, erythrocytes, and leukocytes are especially susceptible to lysis in urine specimens with a low SG (<1.010) and in urine specimens with an alkaline pH (1).

Largely of historical interest, there are few occasions when the color, clarity, and odor of urine are of clinical significance (4). Ammoniacal odors are most commonly due to bacterial degradation of urea, and they can indicate an old specimen or urinary tract infection (UTI). Any unusual physical characteristics should be noted. Intensely colored urines may interfere with proper interpretation of colors formed on dipstick reagent pads (cfr. Infra) (1).

Chemical Urinalysis

Reagent strip, dipstick (syn.) – (Def.) A plastic strip to which is affixed one or various dry chemical reagent impregnated sites (reaction pads) for the qualitative or semiquantitative assay of specific chemical constituents and physical parameters. *Table 1* presents urine test strips' testing principles, indications and pitfalls.

Copied from webpage (5)

Reagent strips for ketone bodies (e.g. acetoacetic acid and/or acetone), albumin, glucose, leukocyte esterase, blood/hemoglobin, nitrite, bilirubin, pH, urobilinogen and pH are commonly available. Following exposure to urine, these reaction pads' color reactions are interpreted visually or evaluated
with a reagent strip reader instrument. A set amount of time is required for the color reaction.

Strips from different lots, let alone from different manufacturers may not be interchangeable. It is important to know the sensitivity and specificity of each test on the strip. Laboratories may wish to conduct its own verification studies (6). Some urine substances can interfere with the chemical reactions. To that end information on prescription and nonprescription medications or dietary supplements (e.g. vitamin C) should be supplied on the urinalysis requisition form (1).

Semiautomated and fully automated reagent strip readers are available that objectively measure the intensity of these reactions and eliminate the variances from reaction-timing and operator-to-operator subjectivity of color interpretation (1,7). Quantitative reading of urinary test strips using reflectometry (*Figure 2*) has become possible, while complementary metal oxide semiconductor (CMOS) technology has enhanced analytical sensitivity and shown promise in microalbuminuria testing (*Figure 1*) (8–10).

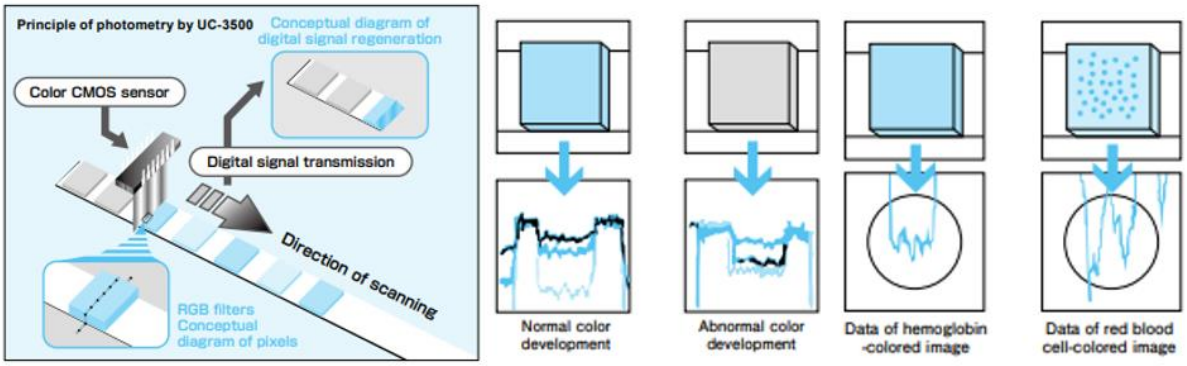


Figure 1: Color CMOS sensors, differentiation of color development.
(Copied from Sysmex Co. Basic Knowledge of Urinalysis. (11)

Urine Concentration (Specific Gravity)

Urine creatinine, specific gravity and osmolality are the most commonly used measurands of urine concentration (12–14). Osmolality is the most accurate assessment of urine concentration, while SG is more easily performed (15,16). Specific gravity of urine is the ratio of the weight of the specimen to the weight of an equal volume of distilled water at the same temperature. Urea (20%), sodium chloride (25%), sulfate and phosphate contribute most of the SG of normal urine (3). All of the methods discussed below are influenced by the number of molecules present, along with their size and/or ionic charge. Most devices determine SG by refractometry.

The hydrometer (urinometer when calibrated for urine), the oldest technique, uses liquid displacement to estimate SG. This device has several disadvantages (need for large volume of urine, glass construction, temperature, inaccurate) and should therefore not be the method of choice (1). Shifts in harmonic oscillation may also be measured and used to calculate relative density. Such measurement offer the advantage of automation and excellent correlation with refractometry, yet require no clarification of cloudy specimens. Refractometers evaluate the SG of a solution by measuring the total dissolved solids in a liquid as indicated by the refractive index of the solution (*Figure 2*) (1). Elevated results can be seen (and corrected) when urine contains x-ray contrast media, plasma expanders, and large amounts of glucose or protein (17). Colorimetric reagent test strips are based on the change in pKa (4). Alkaline urines can affect the indicator system. Strips read instrumentally are automatically adjusted for pH and thus offer the advantage of automation and splendid correlation with gravimetric measurement. In some laboratories, osmometry is performed instead of SG measurement (1).

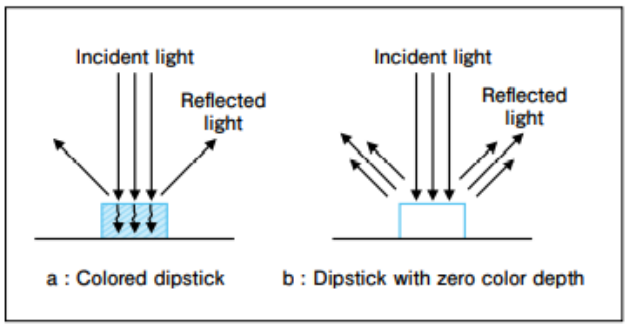


Figure 2: A schematic diagram of the principle of measuring the reflected light (18).

Sediment – Manual Microscopy

Sediment – (Def.) The formed elements of urine that are concentrated by centrifugation or detectable with flowthrough cytometers in a whole urine sample. Elements include cells, casts, crystals, microorganisms and others. *Table 2* presents urine sediment microscopic components' indication, morphology and remarks.

Sediment entities that should be identifiable using a urine microscopic examination include epithelial cells (squamous, renal tubular, transitional epithelial/urothelial), blood cells (erythrocytes [RBC] and leukocytes [WBC]), casts (bacterial, broad, cellular, fatty, granular, hyaline, RBC, waxy, WBC), microorganisms (bacteria, parasites, viral inclusions, yeast), crystals (amorphous, calcium oxalate, cholesterol plates, cystine, triple phosphate, uric acid), miscellaneous (contaminants, mucus threads, sperm) (1–3,19–21). Advanced microscopy skills may be required for the identification of other elements: for instance dysmorphic red blood cells with clinical significance (Figure 3). (*Attachment 1*: Representative photomicrographs of (un)stained elements in urine.) A review of available information, including physicochemical results, is essential before reporting the microscopic examination. Any discrepancies should be resolved.

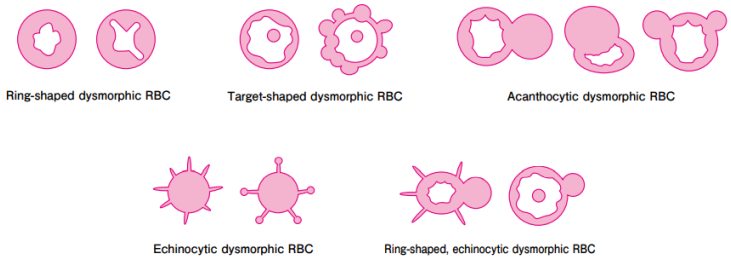


Figure 3: Morphology of glomerular red blood cells (dysmorphic). Copied from (11).

Most urine sediment examinations are done using wet mounts and brightfield microscopy, which is generally sufficient. In some cases, staining may be helpful in the identification on cells and casts (20). The use of phase optics enhances the identification of (abnormal) sediments as is the case for lipids and crystals. (19–24) Disposable, standardized microscope slides or viewing devices with integral calibrated chambers are preferred (Figure 4) in (brightfield) microscopy (1). Though these devices are not amenable to polarization microscopy. Commercial system technologies, which make it possible to report per unit of volume, can provide standardized results. According to the CLSI guideline GP16, the urine sediment is centrifuged for 5 minutes at 400 g (+/- 1500 rpm) (1). In methods where the sample is centrifuged, a variable but significant loss of fragile particles during centrifugation has to be considered (25). The sediment is then examined at a magnification of 400x. The results are expressed as number of cells per field (high power field or HPF). At least 20 fields are examined. Cylinders are best viewed at low magnifications (100x; low power field, LPF). One can also examine urine sediment unconcentrated and count it in a counting room. This technique is very reliable, but very time-consuming (25).

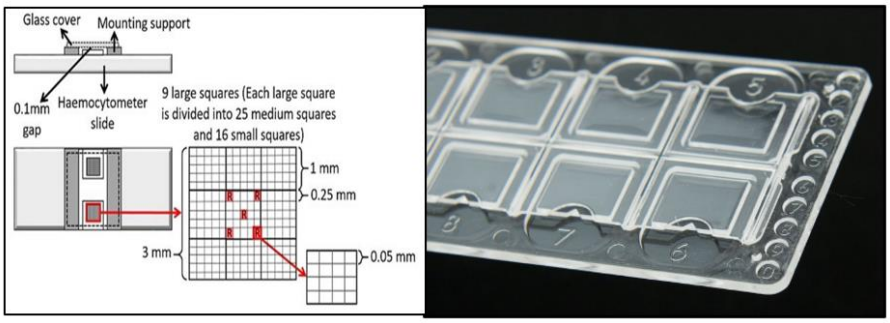


Figure 4: Counting chambers: Improved Neubauer's chamber (left), Disposable Neubauer chamber (right).

Numerous articles address the need for a urine sediment microscopic examination (26–34). The decision to perform microscopy, independently from the physician's request, should be made by the laboratory based on its specific patient population (e.g. with immunosuppressed, renal, diabetic, or pregnant patients), and in case of an abnormal physicochemical result (1,3). Moreover, laboratories performing microscopic urinalysis may wish to develop criteria for identifying suboptimal specimens, based on the presence of common microscopic contaminants indicative of genital/anal contamination (e.g. numerous mature squamous cells, "clue cells", vegetable fibers) in which case the presence of bacteria may not indicate urinary tract infection (1).

Table 1: Semi-quantitative urine test utilizing a urine test strip: testing principles, indications and pitfalls (25,35,36).

Urine component	Testing principle	Indications	Pitfalls
Leukocytes	Leukocyte esterase (lysed WBC) Granulocyte specific	Infections, urinary stones, inflammation	F+: drugs, strongly colored urine (red beets, bilirubin), oxidizing agents, formaldehyde, sodiumazide (preservative) F-: acidic or alkalic pH, ascorbic acid (vitamin C), high protein (>5 g/L) or glucose concentrations, drugs[1]
Nitrite	Nitrate reductase (bacterial)	Infections (*E. coli, Klebsiella, Proteus, Citrobacter*)	F+: strongly colored urine F-: ascorbic acid, alkaline pH, low urinary nitrate, non-nitrite reducing bacteria (*S. saprophyticus, Enterococci, Pseudomonas, Acinetobacter, etc.)*
Erythrocytes / Hemoglobin	Hemoglobin peroxidase (lysed RBC) Differential color development due to hemoglobin or intact erythrocytes	Hematuria due to kidney damage (e.g. glomerulonephritis), infection, kidney or bladder stones, malignancies, or blood disorders	F+: myoglobinuria (due to muscle damage), oxidizing agents, bacteria F-: inability to hemolyze RBCs due to acidic or alkaline urine, ascorbic acid, high nitrite concentration, high urine density, formaldehyde
Protein	Presence of albumin (pH indicator error)	Kidney damage, harmless physiological phenomena (posture-related, exercise)	F+: alkaline pH, drugs, heavily pigmented urine, drugs[2], contrast media F-: albumin concentrations below 300 mg/L, microproteinuria, tubular protein, Bence Jones-proteinuria
Glucose	Glucose oxidase/peroxidase	Glucosuria (tubular reabsorption limit in young adults ~ 1.8 g/L), renal diabetes	F+: oxidizing agents F-: ascorbic acid, UTI, acidic urine (keto acidosis, aspirin usage), reducing sugars (galactose, fructose, etc)
Ketones	Legal reaction (Acetoacetate, acetone)	Fatty-acid oxidation (ketosis), ketoacidosis (diabetes, chronic alcoholism, etc.), physiological (exercise, fasting)	F+: drugs[3] F-: pre-analytical storage
pH	Universal pH indicator	Kidney or urinary tract disorder	Alkaline pH due to bacterial growth (bacterial urease), dietary (vegetables), Fanconi syndrome (aminoaciduria), cast-forming due to alkalic urine Acidic pH due to dietary (meat, cranberries)
Urobilinogen	Ehrlich reaction	Impaired liver function, increased hemoglobin degradation (hemolytic anemia)	Decreased urobilinogen may indicate a blockage in the bile duct system or bile production failure.
Bilirubin	Ehrlich reaction	Hemolysis, liver damage or disease (jaundice).	F+: rifampicin
Creatinine	Benedict-Behre method	Kidney diseases	F-: ketone bodies, ascorbic acid (> 200mg/dL)
P/C **A/C**	Protein/Creatinine ratio Albumin/Creatinine ratio	Higher sensitivity for A/C ratio than conventional protein dipstick.	Albumin dipstick ~ 10-150 mg/L
Specific gravity	Refractometry	Urine concentration	F+: intravenous contrast media

F+: false positives, F-: false negatives.
[1]Cefalexine, cephalothin, nitrofurantoin, tetracycline, tobramycin.
[2]Penicilline, cephalosporins, miconazole, tolbutamide, sulphonamide metabolites.
[3]Phenylketones, phthalein derivatives, levodopa metabolites, captopril, sulfhydryl-containing components.

Table 2: Urine sediment microscopic analysis: Component's indication, morphology and remarks (10,25,35,36).

Urine component	Indication	Morphology	Note
Erythrocytes	Kidney disease, a blood disorder or another underlying medical condition, such as bladder cancer Temporary erythrocyturia in children not uncommon (unsignificant)	Tonicity of urine: - Echinocyte or burr cell (hypertonic), - Bloating or lysis (hypotonic) Glomerular and non-glomerular hematuria: - Dysmorphic; acanthocyte (renal hematuria; glomerulonephritis), - polymorphic (urologic hematuria)	Lysis due to sample freshness, alkaline pH, low osmolality, casts due to glomerulonephritis
Leukocytes	Infections (UTI)	In conjunction with possible microorganisms. Glitter cells in hypotonic condition; polymorphonuclear neutrophils with granules showing a Brownian movement. Eosinophils in drug induced interstitial nephritis.	Casts due to UTI Sterile pyuria exists in kidney tuberculosis, polycystic kidneys, malignancies Eosinophils require staining (Hansel)
Epithelium	Physiological conditions UTI, inflammation Kidney damage	Superficial urothelium : squamous epithelium Deeper layers urothelium: 'small round cells' Tubular epithelium	
Casts (cylinders)	Kidney disorders, physiological conditions	Hyaline casts: - consist of Tamm-Horsfall protein secreted by urothelium, can be exercise-related Cellular casts: - kidney pathology; erythrocyte cast (glomerulonephritis), leukocyte cast (pyelonephritis) Granular casts: - kidney disorder; due to autolysis (granulation) Wax casts: - severe chronic kidney disease (diabetic nephrosclerosis, nephrotic syndrome); denaturation of plasma proteins in tubuli (associated with proteinuria)	Fragile and brittle particles
Crystals	Kidney stones: trivial, pathological, drug induced	- Trivial crystals and amorph deposits (calcium oxalate, urate, phosphate) - Drug induced: e.g. indinavir, sulfamethoxazole-trimethoprim, ciprofloxacin - Pathological: cystinuria (hexagonal), xanthine, leucine, tyrosine	Diuresis, dietary, urinary pH
Oval fat bodies	Lipoid nephrosis	Oval fat bodies due to leakage of plasma lipoprotein Cholesterol crystals (polarization microscopy)	Also isolated small fat droplets in sediment
Mucine threads	Physiological condition	Urothelium coated with mucin threads	
Organisms	Bacteria, yeast, fungi, parasites	Infections	Contamination, worm eggs

Automated Urine Particle Analysis

In recent years, automated urine testing has been developed. Automated urinalysis systems are designed to provide maximum convenience to the user, enhance productivity, and eliminated specimen preparation (1,7,37). Two types of systems are currently available on the market: urinary flow cytometry (UFC) and automated microscopic pattern recognition. Semiautomated urinalysis instruments that perform microscopic examination can quantify and standardize urine sediment. It automatically classifies formed elements for confirmation by a qualified operator, which provides increased sensitivity and reproducibility and ease of use (8). A key advantage is the larger number of sediment elements actually enumerated, as compared with manual microscopy (37–40). However, automation may have limitations in patient populations with a high prevalence of nephropathology (41). With these techniques, the required sample volume is low and the statistical counting error is lower than with a classical sediment (25). Though, various technologies are used to classify and enumerate particles in urine. Depending on the instrument, the technologies incorporated can include impedance, digital imaging, flow cytometry, light scatter, dyes, fluorescence, or some combination thereof. Particles with like characteristics are then grouped and classified. The elements that are typically identified include RBC, WBC, epithelial cells (squamous and non-squamous), casts (benign and pathologic), bacteria, sperm, mucus, crystals, and small round cells.

A common approach is to combine test strips with UFC for primary screening. Expert systems were developed to verify results and reduce errors. Samples showing abnormalities, either laboratory-defined or as indicated by the analyzer, are then reviewed microscopically by a technologist. Visual microscopy thus remains necessary (reviews). For example, for differentiation of cylinders (or casts), tubular epithelial cells, dysmorphic erythrocytes, fungi, Trichomonas and other parasites, and clinically significant crystals (25). Negative samples may obviate the need for manual microscopy (8,41–45). Ongoing morphologist competence assessment should include workshops, seminars, self-study programs, proficiency surveys, comparison with other microscopists, internet-based systems, etc (46,47).

Urine Flow Cytometry

> *"Cytometry, now broadly defined to include counting, classification, and characterization of biologic cells and similarly sized objects, began when cells were first discovered in the late 1600s. The discovery of pathogenic bacteria and of disorders such as anemias, leukemias, and malaria, in which cellular changes in the blood could be correlated with clinical course, brought microscopy into clinical use in the mid-1800s. Until about 1950, cytometry depended on human observers using microscopes. From then on, increasingly sophisticated instruments known as cytometers have replaced microscopy wherever budget and infrastructure allow. Cytometry is a complex technology, but the most complex cytometers, although considerably larger, are much simpler than the simplest cells."*
>
> Shapiro HM. Cytometry in: Rifai's *Tietz Textbook of Clinical Chemistry and Molecular Diagnostics*. (48)

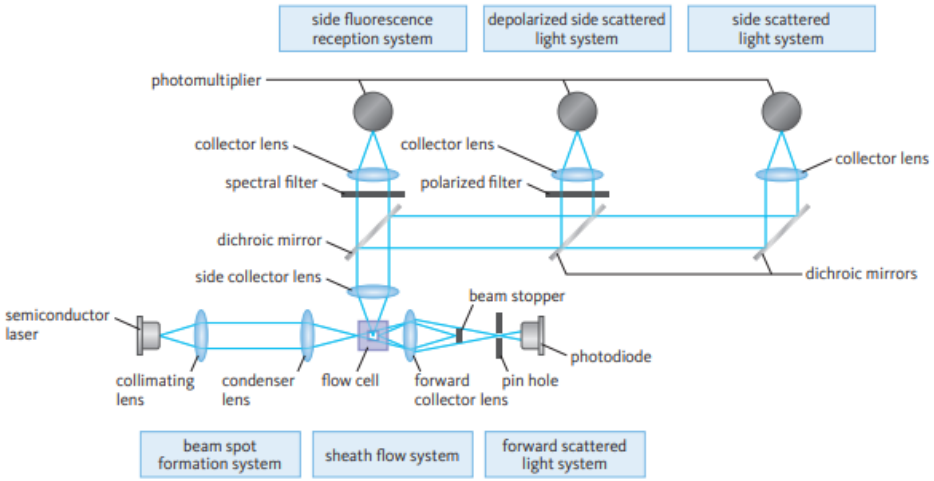


Figure 5: Flowcytometer urine particle analyzer technical set-up.

The flow cytometer counts red blood cells, white blood cells, bacteria, yeast cells, crystals, squamous epithelial cells, small round cells and sperm cells. The particles are coloured with fluorescent dyes or stains. There are two types of stains (Fluorocell, Sysmex), both with polymethine dye as the main component (49). Lipids, certain crystals, casts and epithelial cells are not recognised (transitional epithelial and renal tubular cells are classified as small round cells). Particles that cannot be classified into any of the above categories are counted as 'other cells' (25). Number of both reportable and research parameters have been increased through the use of new measurement principles and reagent. Cytometers may measure different properties (or, to use standard cytometric jargon, “parameter”) of each cell analyzed (48).

One stain is for the SF channel (*surface*) that analyzes particles that do not contain nucleic acids such as red blood cells, casts and crystals. It uses waveform information about particles, in addition to the scattered light intensity, signal width. The diluent Cellpack-SF dissolves amorphous salts and disperses mucus, both of which interfere with the measurements. The stain Fluorocell-SF then stains the membrane components (RBCs, cast matrix, etc.).

Another, the CR channel (*core*) analyzes nucleic acid-containing particles such as white blood cells, epithelial cells, yeast-like cells, spermatozoa and bacteria through the combined use of multiple characteristics like scattered light signal width, scattered light signal waveform area and fluorescent signal waveform area. The diluent Cellpack-CR lyses or dissolves RBCs and crystals, which can interfere with the classification of particles. On the other hand, WBCs and epithelial cells are not lysed. The surfactant in this diluent creates fine pores on the cell membranes and the polymethine dye in Fluorocell-CR can thus enter to stain nucleic acids in the cells.

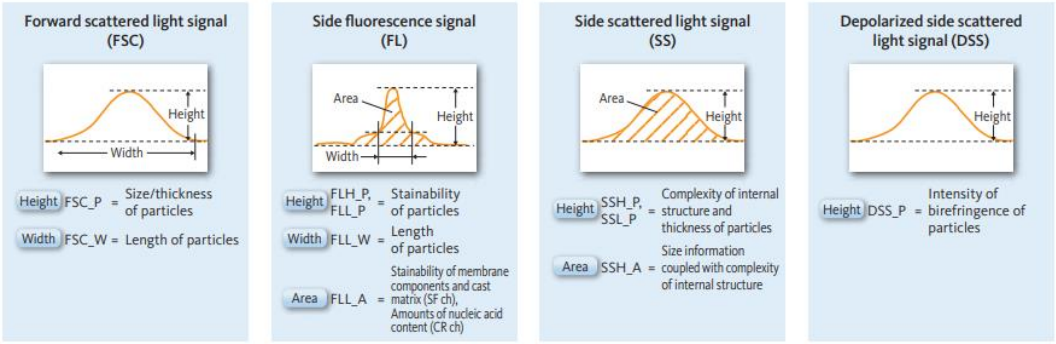


Figure 6a: Signal waveforms obtained from light scattering, fluorescence signals and impedance.

The sample is then delivered to a flow cell using a sheath flow technique to ensure that a single-object stream passes through the flow cell (hydrodynamic focussing). The laser beam is aimed in a direction perpendicular to the flow of the sample. A blue semiconductor laser beam is irradiated on particles stained with a Fluorocell stain and the particle is then classified based on four types of signals.

Scattered light and fluorescence are detected by a photo diode and converted into electric signals. Forward scattered light, which mainly reflects information about the size and permeability of particles. Side scattered light, which reflects the thickness and internal structure of particles. Fluorescence intensity, for the stainability of particles. And depolarized side scattered light in consideration of the intensity of birefringence of particles (*Figure 5*), which was introduced to improve crystal sensitivity and to better discriminate between RBCs and crystals (10). Waveform processing analysis is conducted on these electric signals. The following waveforms are obtained from the four types of signals (*Figure 6a and 6b*). (10,37–39)

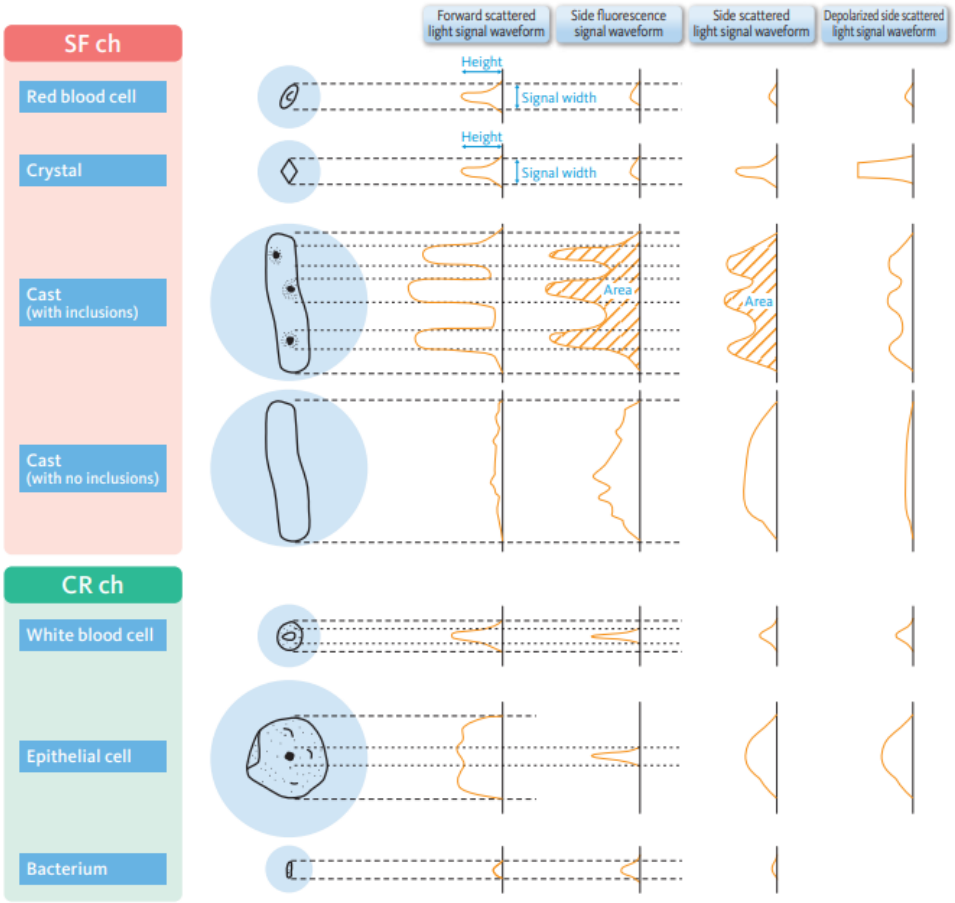


Figure 6b: Signal waveforms obtained from light scattering, fluorescence signals and impedance.

Two-dimensional scatter diagrams of the combination of these parameters are automatically created and used to classify and quantify the different particles (39). Scattergram patterns illustrate the location of particle clusters (*Figure 7-10)*.

Diagrammatic illustrations of SF channel (*Figure 7*)

Red blood cells (RBC) appear in the low intensity zone of depolarized side scatter. Non-lysed RBCs are the ones with relatively high intensity forward scatter and are considered morphologically stable. Glomerular and non-glomerular RBCs are thought to be included. Lysed RBCs have opposite characteristics. Glomerular RBCs or degenerated RBC with unknown origin are included in the latter. The analyzer assesses research information from the distribution of the RBC histogram whether the RBC morphology is normal (isomorphic or dysmorphic). However, when total RBC counts are below a certain threshold (e.g. 20 /µL) or in case of low reliability, the message is not displayed.

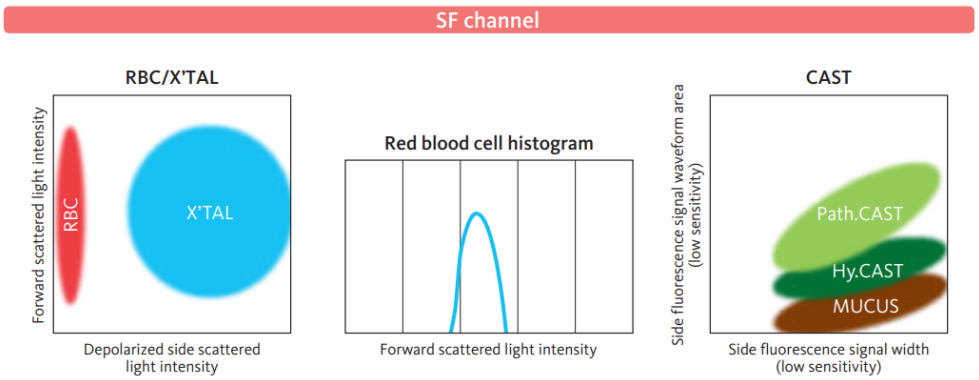


Figure 7: Diagrammatic illustrations of SF Channel.

Differentiation of red blood cells from crystals (X'TAL) has been significantly improved by detecting birefringence, which is a characteristic of crystals by the use of depolarized side scattered light. The plane of polarization twists or rotates when the blue laser hits the crystal. Light then passes through a polarized filter in front of a photomultiplier.

The side fluorescence area reflects the stainability of cast matrix and the width reflects the length of the cast. Mucus is dispersed by the surfactant contained in Cellpack-SF. Pathological casts (Path.CAST) contain a larger number of inclusions and so its side fluorescence area is larger than that of the hyaline casts (Hy.CAST) (Figure 8).

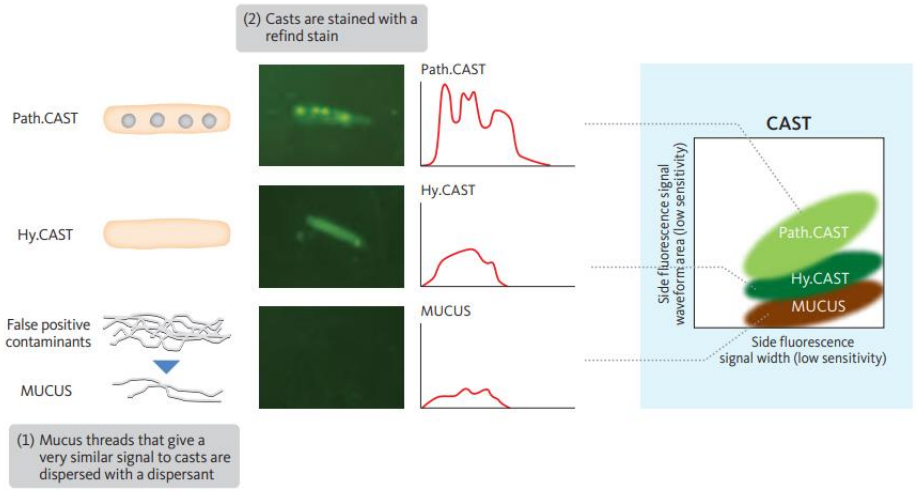


Figure 8: Scattergram pattern for Cast information.

Diagrammatic illustrations of CR channel (*Figure 9*)
White blood cell (WBC) clumps have a larger side fluorescence signal waveform than WBC because of a higher nucleic acid content. As well as a higher forward scatter width due to larger size. When a bacterial urinary tract infection is suspected it is displayed as research information based on the combination of WBC and bacteria count. Threshold values whereupon triggers are set can be changed.

Debris (DEBRIS) are fine components such as cell fragments and are separated from bacteria.

The bacteria (BACT) scattergram displays research information on Gram-staining estimated from the scattergram. Though, the analyzer does not make this assessment when the BACT-count or WBC-count is below the threshold values. The assessment is based on the known differences in composition of the cell wall. Using forward scatter to reflect the peptidoglycan layer and side fluorescence to reflect the amount of dye that has penetrated into the bacterial cell (*Figure 10*).

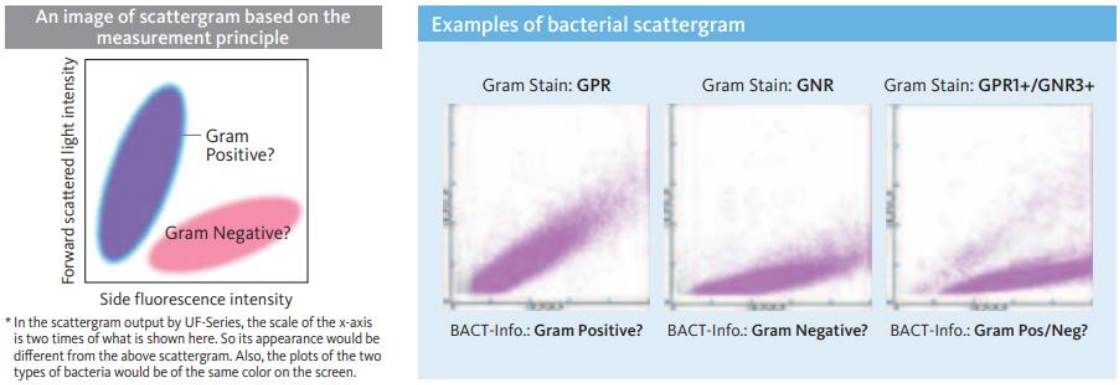


Figure 10: Scattergram pattern for Bacteria Research Information.

Yeast-like cells (YLC) may have several nuclei depending on the state of budding. The size of the cell (forward scatter) and amount of nucleic acid (side fluorescence) increase as budding progresses further and this leads to the distribution of the dots spreading diagonally upwards towards the right on the scattergram.

The heads of spermatozoa (SPERM) are uniform in size. Therefore, forward scatter and staining intensity (side fluorescence) are constant.

Squamous epithelial cells (Squa.EC) consist of low amounts of nucleic acid content and the size information coupled with complexity of internal structure is small relative to the length of particles. Therefore, their side fluorescence area and side scatter area are relatively smaller with respect to their forward scattered width, which reflects the length of the particles. Non-squamous epithelial cells (Non-SEC) are further classified into transitional epithelial cells (Tran.EC) and renal tubular epithelial cells (RTEC). Both appear in the same area of the scattergram using the length of particles (forward scattered light signal width) and the amounts of nucleic acid content (side fluorescence signal waveform area) or the length of particles and the size information coupled with complexity of internal structure (side scattered light signal waveform area). This principle is based on that Tran.EC tend to have a greater amount of nucleic acid content than RTEC for a given cell size. Small round cells (SRC) are the same as RTEC and are included as a research parameter.

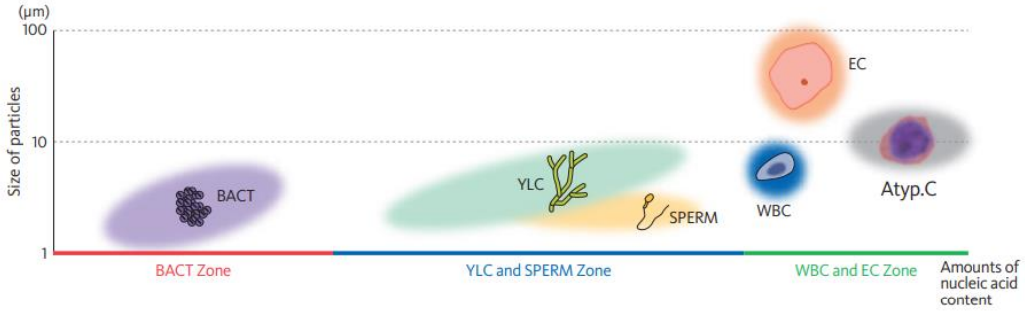


Figure 11: Nucleic acid containing particles detected in the CR channel.

Atypical cells (Atyp.C) can also be classified as a research parameter based on forward scatter width (length) and side fluorescence area (nucleic acid content). It includes all particles having large side fluorescence area such as atypical cells, cells with cytoplasmic inclusions, and virus infected cells (Figure 10).

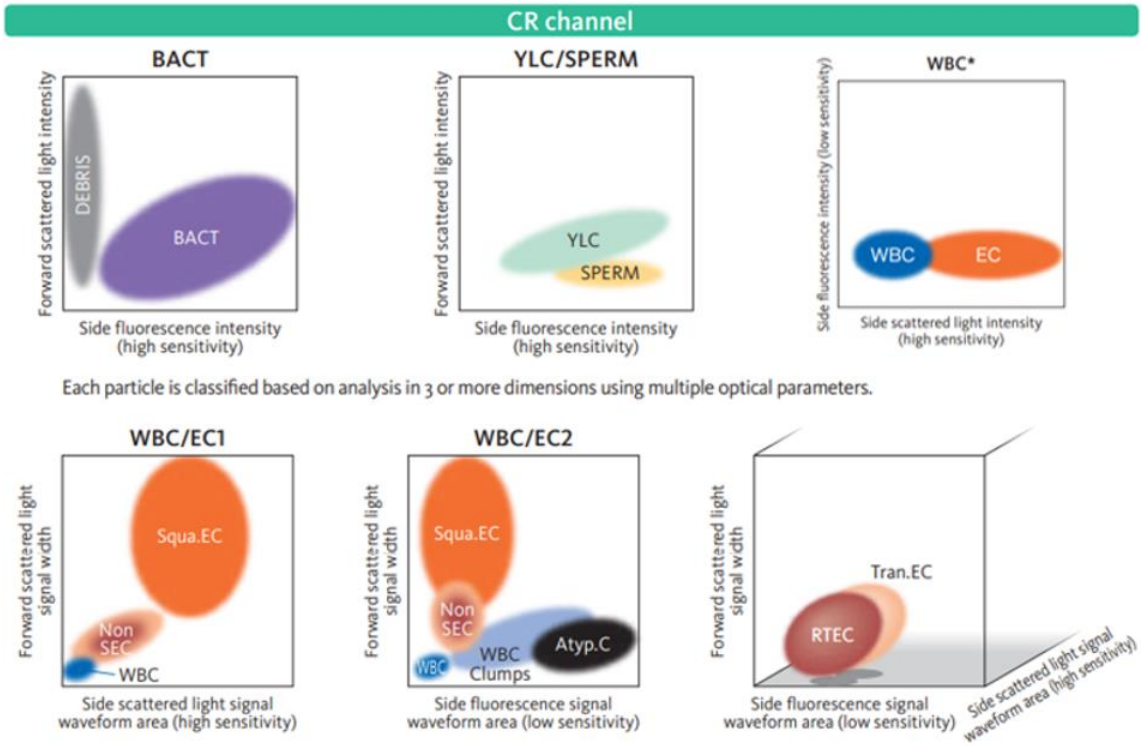


Figure 9: Diagrammatic illustrations of CR Channel.

Remarks on Urine Flow Cytometry (Discussion)

Urinary flow cytometry technology has been improved to the point where it may be considered a next generation urine particle analyzer. With future advancement of clinical application, such as direct comparison of information obtained by the analyzer with guideline-based clinical disease profiles, analyzers are expected to provide new information and not be merely positioned as a screening device before deciding to undertake microscopic urine sediment analysis (50). Studies demonstrate the significant decrease in samples requiring microscopy even in laboratories already analyzing only a minor percentage of samples via microscopy before introduction of automated systems (43). Flow cytometry allows precise and accurate counting, as well as acceptable linearity over clinically useful working ranges (10). The quality of the analysis is better than that of a manual count, with an imprecision that is consistently and significantly less than that of microscopy and with negligible carry-over (39). And in contrast to classical sediment analysis, fragile particles (e.g. casts) are not damaged.

Multiple studies have put urinary flow cytometry to the test by comparing it with chamber counts, quantitative urine microscopy, sediment counts, test strips, bacterial culture, and urine density (43,51,52). Automated urinalysis can therefore be used for urinary tract infection screening and for diagnosing and monitoring a broad variety of nephrological and urological conditions (10). The bacteria scattergram can preliminary be applied in discriminating UTIs caused by either gram-positive or gram-negative bacteria, or both (53,54). Other studies show a reduction of bacteria cultures of up to 75%, depending on what ratio of false negatives is acceptable (an efficient screening method) (55–59). Some illustrate how reliable the automated analyzer can classify actual patient samples, demonstrating a performance similar to that of CLED agar cultures and an excellent negative predictive value (NPV) surpassing bacterial culture (60). In using test strips (leukocyte esterase, nitrite, urinary protein and hemoglobin) and microscopic examination (either manual or automated: WBCs, microorganisms), others demonstrate such screening-out methods to be rudimentary, subjective, time-consuming and having poor NPV (61). In addition, discrepancies in bacterial counts between automated counting and culture occur where bacteria can be found in urine that are dead or growth-impaired (37). The first factor for divergent results is detection and counting of viable and dead bacteria due to the characteristic of the reagent (39,62). The second confounding factor may be so-called viable but non-culturable conditions under which the bacteria cannot grow on agar (63). But overall good correlation with the cultural method can be obtained. Accordingly, the applicability of flow cytometry to screen for negative urine samples strongly depends on population characteristics and the definition of a negative urine culture (58).

Generally speaking studies using urinary flow cytometry have focused on diagnosis and evaluation of urinary tract infection, localization of the origin of hematuria, and monitoring or exclusion of renal diseases. A practical and commonly used approach is to combine test strips with flow cytometry for screening, or by utilizing the strips for measurands unrelated to the flow cytometry-particles. It should be noted, however, that (digital) microscopy continues to have a place in urine diagnostics. As discussed earlier on, numerous articles address the need for a urine sediment microscopic examination. The decision to perform microscopy, independently from the physician's request, should be made by the laboratory. In case of samples showing abnormalities, either laboratory-defined or as indicated by the analyzer, visual microscopic review remains necessary. Definitely in consideration of parameters that can't be identified unambiguously using flow cytometry; for instance differentiation of casts, non-squamous epithelial cells, dysmorphic erythrocytes, fungi, parasites and clinically significant crystals. As automation continues, expert systems have been developed to combine all complementary test modalities based on user-definable decision-rules, which improves the quality of the test results (43). An implementation of this kind has the ability to reduce microscopic reviews and save time and cost (workload, unnecessary empirical antibiotic prescriptions) without compromising on clinical applications (53,54,58,61,64–73).

Conclusion

Improvements and Innovation in Urinalysis

Urinalysis, one of the oldest medical techniques, has arrived in the 21st century. With the introduction of automated analytical systems, solutions have been developed to automate the counting of particles in urine. Multiple goals are pursued in this development. To reduce the timeframe in which results can be expected (turnaround time, TAT) and thus avoid treatment in cases in which it is not needed. The time-saving effect also frees resources for additional samples, thus increasing the daily throughput of the laboratory. Further, to free the highly qualified personnel for cases in which their expertise is actually needed instead of occupying them with readily distinguishable negative cases. And to allow the generation of reproducible results with standardized procedures. Despite improvements in standardization, errors occur outside of the analytical phase; pre-analytical conditions in particular are much more of a liability.

Quantitative reading of urine strips using reflectometry and CMOS technology is available. Integration of urine concentration parameters allows for correction for urinary dilution. While dipstick test strip analyzers can apparently offer (similarly) quick results, they detect only a certain subset of diseases and infections. To this end, remarkable technical progress can be based on flow cytometry, along with automated microscopic particle analysis. (*Attachment* 2: Scattergram diagrams and corresponding photomicrographs elements.) The combined analysis of scattered light and fluorescence allow the rapid identification and differentiation of particles such as leukocytes, bacteria and even iso- or dysmorphic red blood cells. Besides, it allows a reproducible assessment of particle concentration in urine and consequentially a reliable screening procedure for urinary tract infections and hematuria cases. Along with other more arcane parameters like epithelial cells, spermatozoids, casts, crystals and even atypical cells. The added value and complementarity of a digital microscope for qualitative microscopic reviews on indication becomes clear. The sample throughput of such analytical systems combined with the experience in fluorescence flow cytometry and digital microscopy can contribute greatly to the laboratory workflow.

"Some astronauts describe the routine flushing of urine into space,
where the freezing temperatures turn the droplets into a cloud of bright, drifting crystals,
as being among the most amazing sights they saw on an entire voyage."

Eugene Cernan†, Astronaut and Last Man on the Moon (as of 2022)

TO DO/ACTIONS

1) Validation and implementation of the new urinalysis analyzers UC-3500, UC-1000, UF-5000 and UD-10.

   - As with any automated clinical laboratory method, the laboratory should verify performance of attributes such as accuracy, precision, analytical specificity, interfering substances and measurement range (CLSI documents EP05, EP09, EP17, EP10 and EP15) (1,74–78).

2) Determination whether the new urinalysis analyzer UF-5000 can be used as a screening alternative to rule out an UTI in an early-stage.

   - Suggestion to follow up on the basis of:
     - CAT "To culture of not to culture a urine sample: that's the question".
     - EP12-A2: User protocol for Evaluation of Qualitative Test Performance; Approved Guideline.

3) Reviewing the current modality of requesting and reporting urine diagnostics..

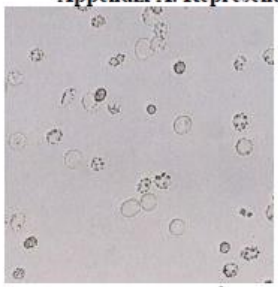
   - At present, a test strip in itself is not reimbursed by the social security system (RIZIV). Since the test strip inherently harbours many pitfalls, it is always advisable to combine both techniques of test strip and sediment analysis, and thus request them together. Especially with the additional flow cytometric technique, which completes the picture of urinalysis.

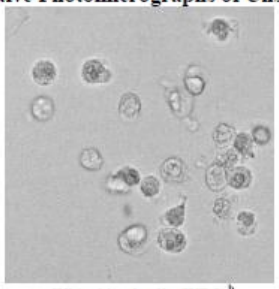
     The reporting of urine parameters should also be reviewed. In case of a trivial urinalysis request, only RBC and WBC concentration and test strip results could be reported. Any abnormalities: e.g. non-pathological casts and/or crystals, bacteria, yeast cells; should not be reported (if not clinically suggestive). Only in cases of nephrological, urological or other special conditions should the full range of microscopically examinable particles be reported.

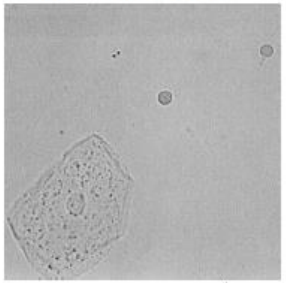
ATTACHMENTS

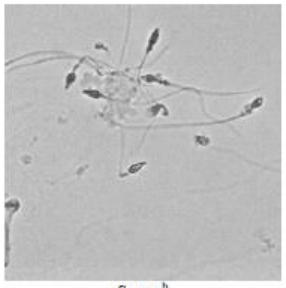
Attachment 1: Representative photomicrographs of (un)stained elements in urine (*Published in CLSI Document GP16-A3.* (1))

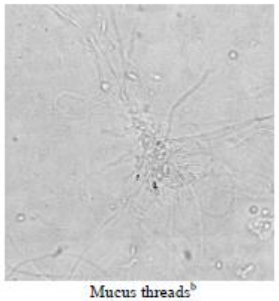
Appendix A. Representative Photomicrographs of Unstained Elements in Urine

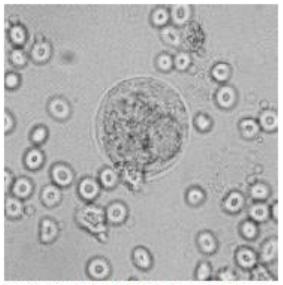

Red blood cells (RBC)[a]

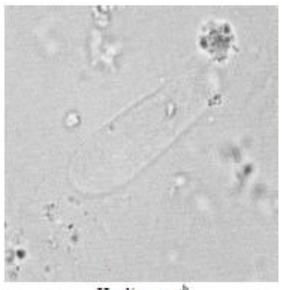

White blood cells (WBC)[b]


Squamous epithelial cell[a]

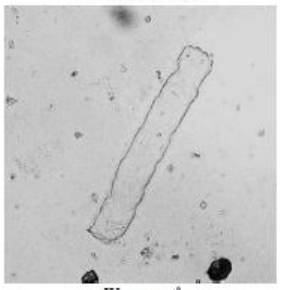

Sperm[b]


Mucus threads[b]


Viral inclusion bodies in macrophage or epithelial cell[a]

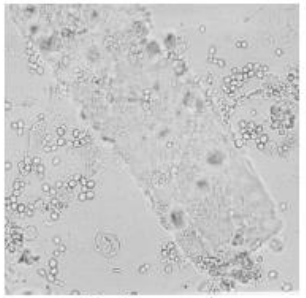

Hyaline cast[b]

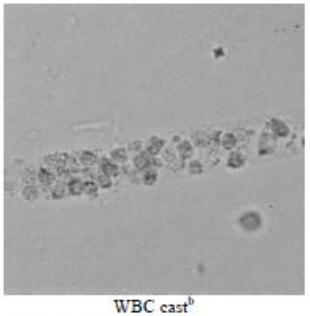

Granular cast[a]

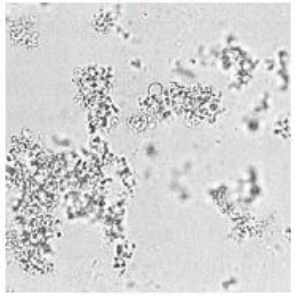

Waxy cast[a]

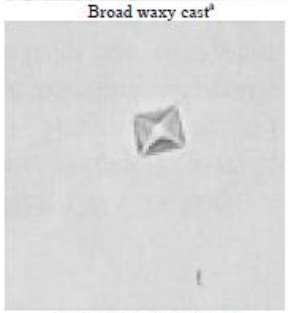
Appendix A. (Continued)


Cellular cast[a]

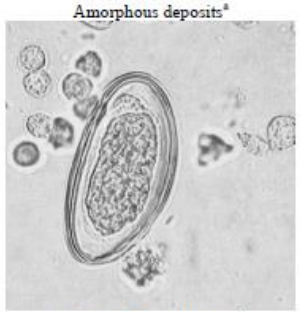

Broad waxy cast[a]

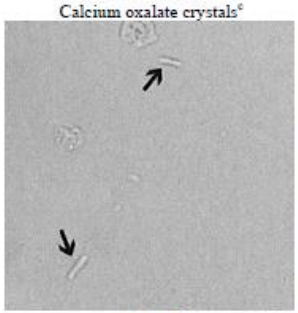

WBC cast[b]


Amorphous deposits[a]


Calcium oxalate crystals[c]


Uric acid crystals[a]

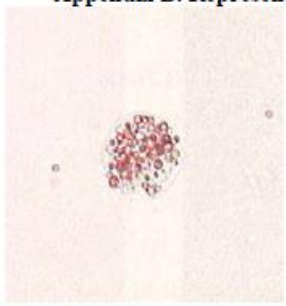

Enterobius vermicularis[c]


Bacteria[a]

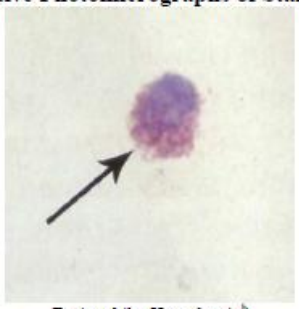

Yeast and WBC[d,e,f]

NOTE: The images in Appendix A represent examples of the types of cells that are commonly seen in urine. This document is not intended for use as an atlas of morphology.

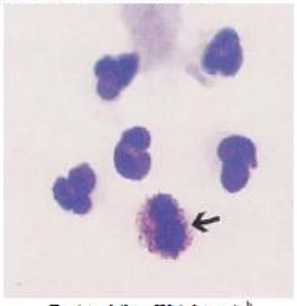
Appendix B. Representative Photomicrographs of Stained Elements in Urine



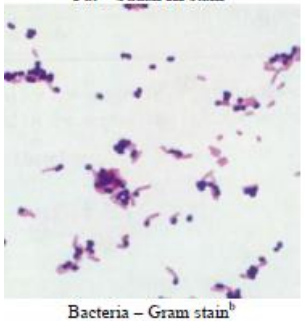
Fat – Sudan III stain[a]



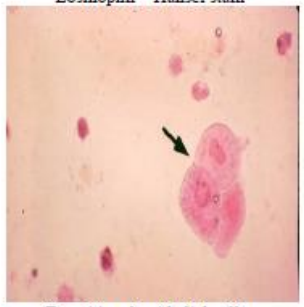
Eosinophil – Hansel stain[b]



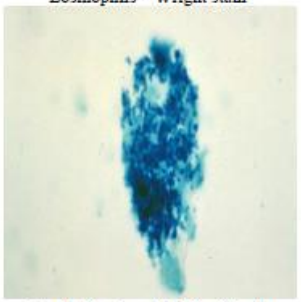
Eosinophils – Wright stain[b]



Bacteria – Gram stain[b]



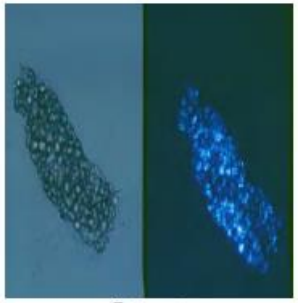
Transitional epithelial cells – Sternheimer-Malbin stain[c,d,e]



Renal tubular epithelial cell with hemosiderin – Prussian blue stain[c,d,e]

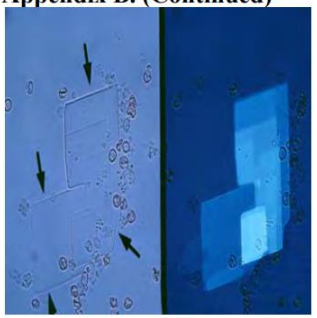
RBC cast[c,d,e]

Fiber, exogenous[d,f,g]

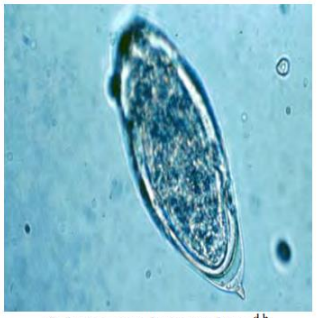


Fatty cast (brightfield and polarized)[d,f,g]

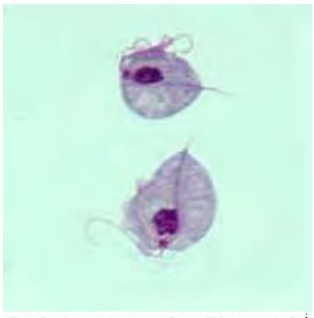
Appendix B. (Continued)



Cholesterol plates (brightfield and polarized)[d,f,g]



Schistosoma haematobium[d,h]

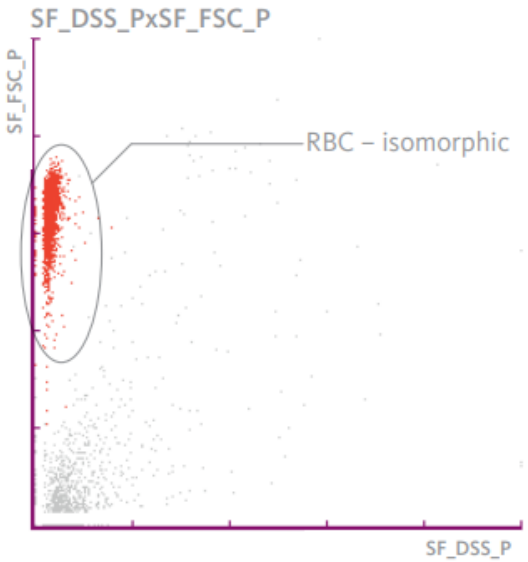


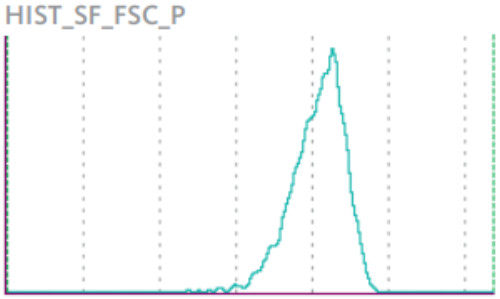
Trichomonas vaginalis – Giemsa stain[i]

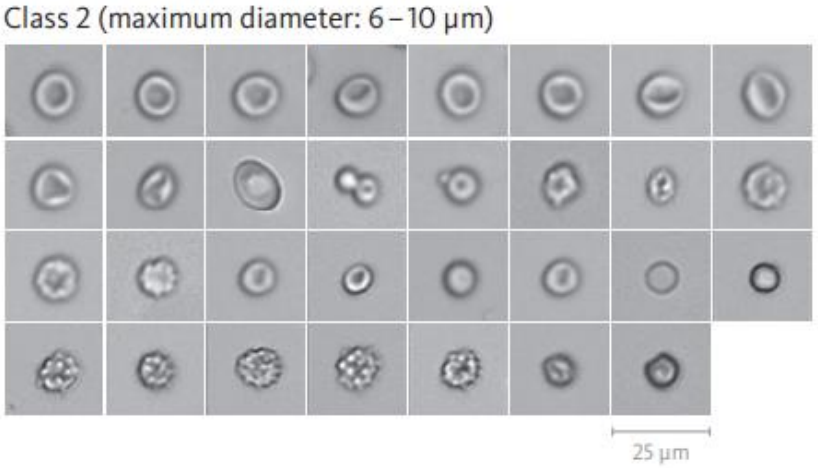
NOTE: The images in Appendix B represent examples of the types of cells that are commonly seen in urine. This document is not intended for use as an atlas of morphology.

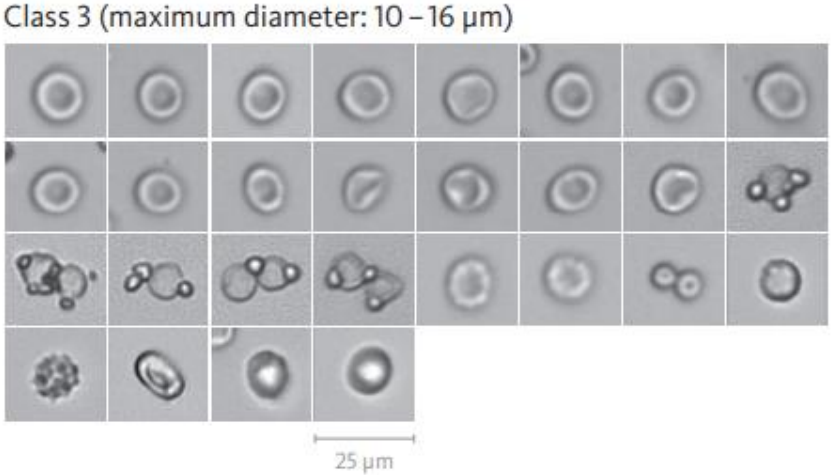
Attachment 2: Scattergram diagrams and corresponding photomicrographs elements (*Copied from UN-Series: Urine Particle Atlas.* (79))

- Red blood cells
    - Isomorphic

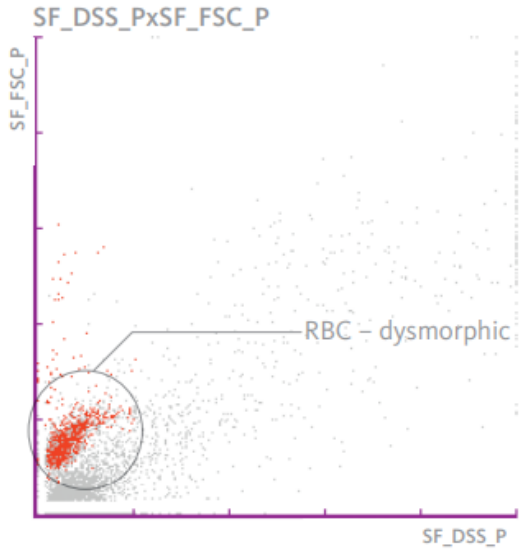


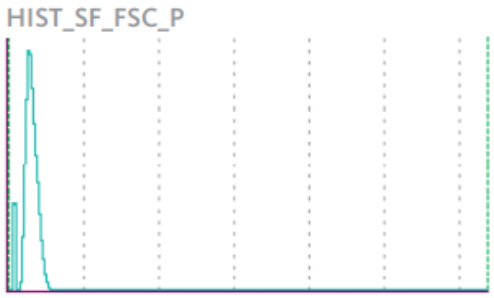


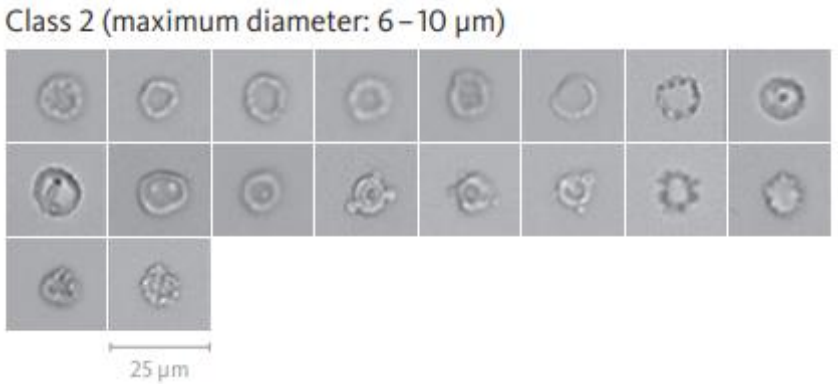


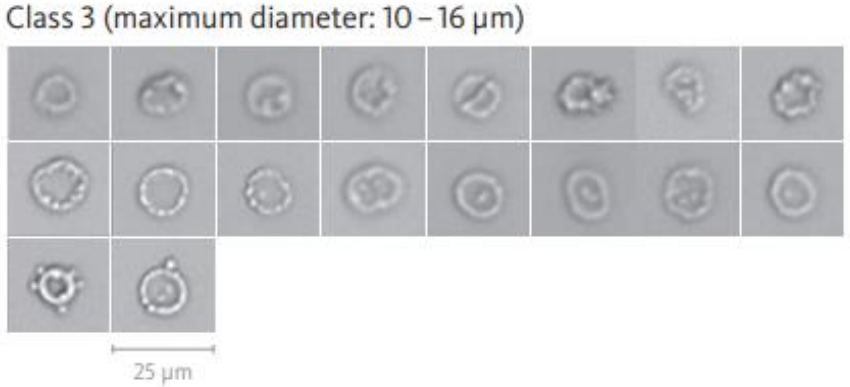


- Red blood cells
    - Dysmorphic

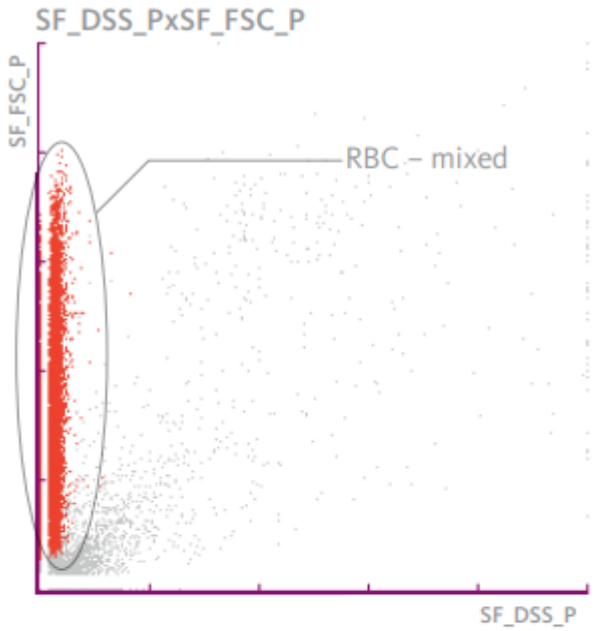


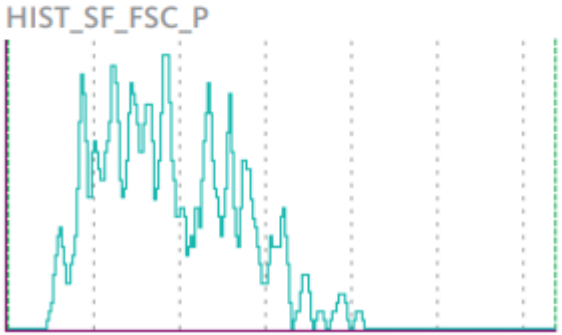


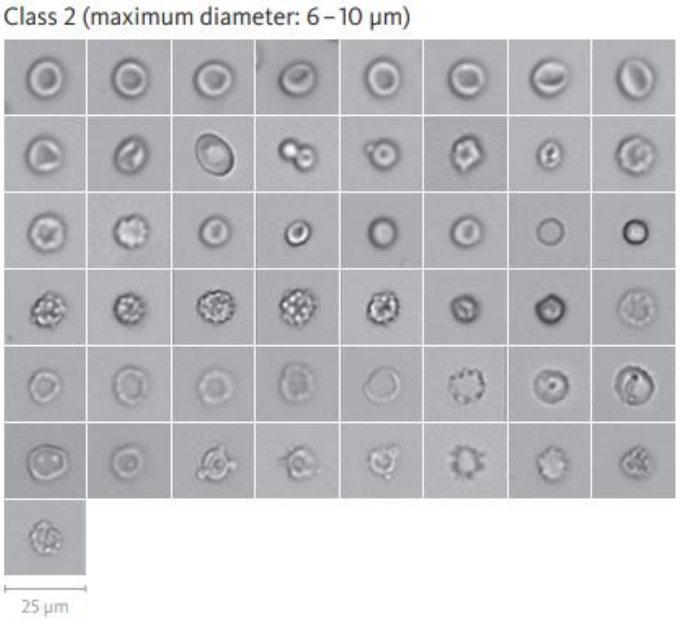


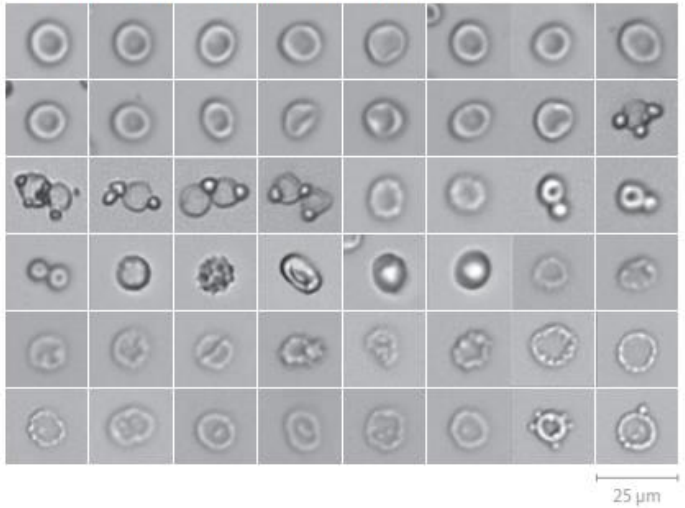


- Red blood cells
    - Mixed



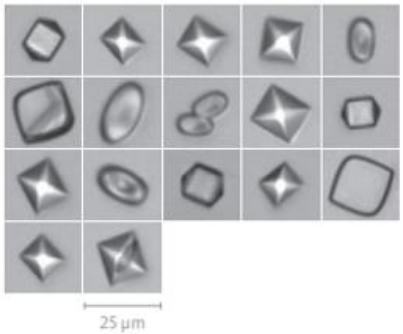




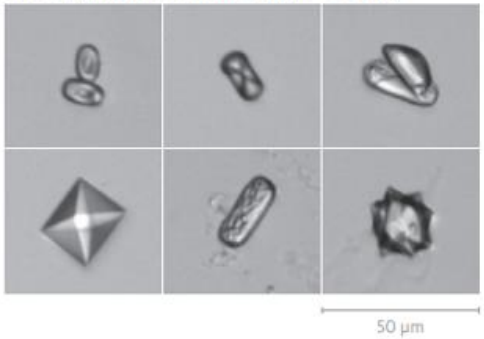


- Crystals

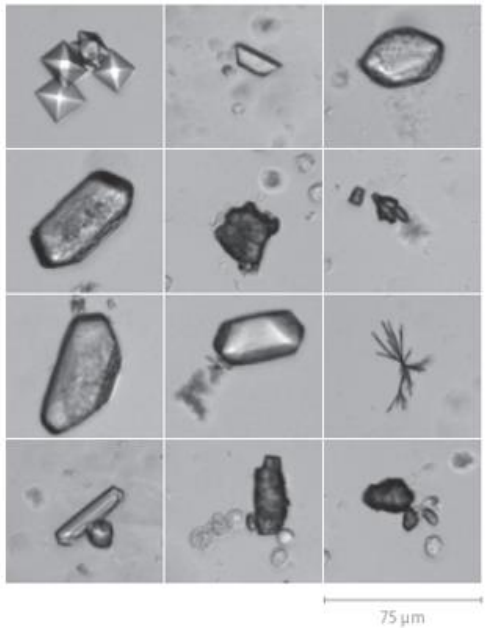
Class 3 (maximum diameter: 6 – 10 µm)



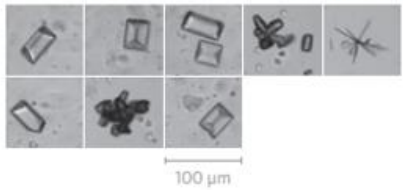
Class 4 (maximum diameter: 16 – 36 µm)



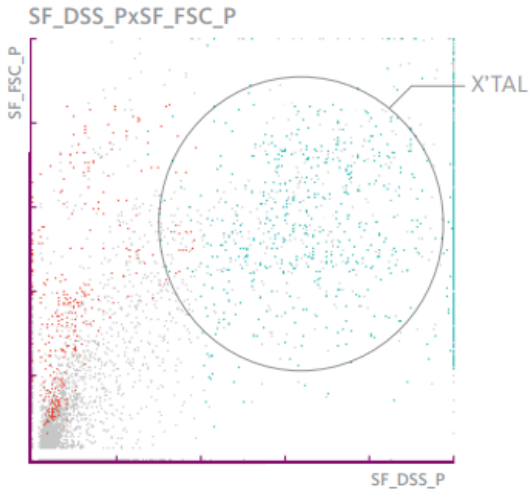
Class 5 (maximum diameter: 36 – 71 µm)



Class 6 (maximum diameter: 71 – 101 µm)

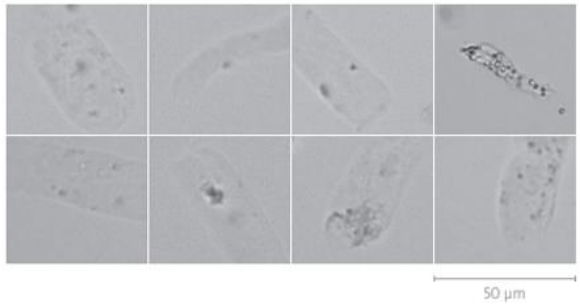


Class 7 (maximum diameter: 101 – 151 µm)

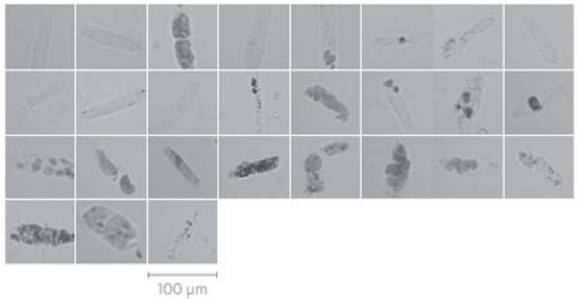


- Casts

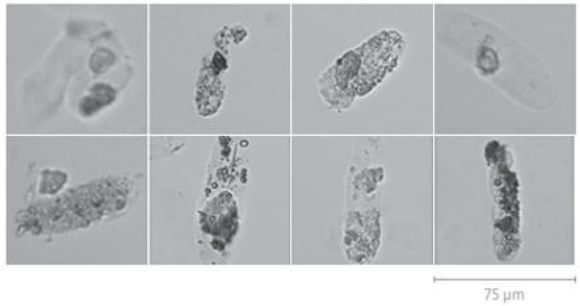
Class 4 (maximum diameter: 16 – 36 µm)



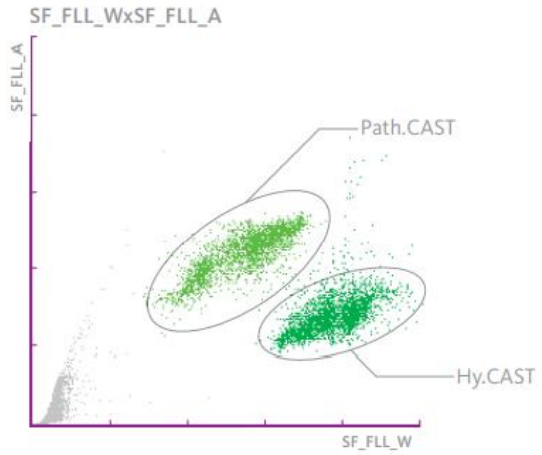
Class 6 (maximum diameter: 71 – 101 µm)



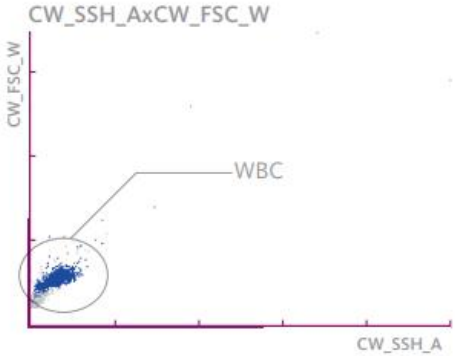
Class 5 (maximum diameter: 36 – 71 µm)

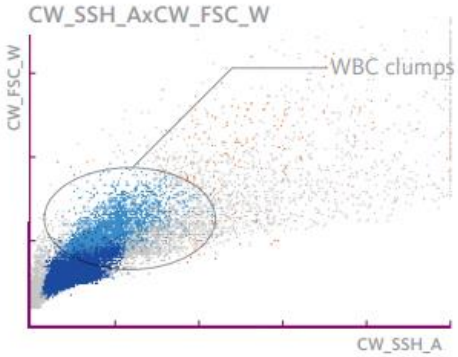


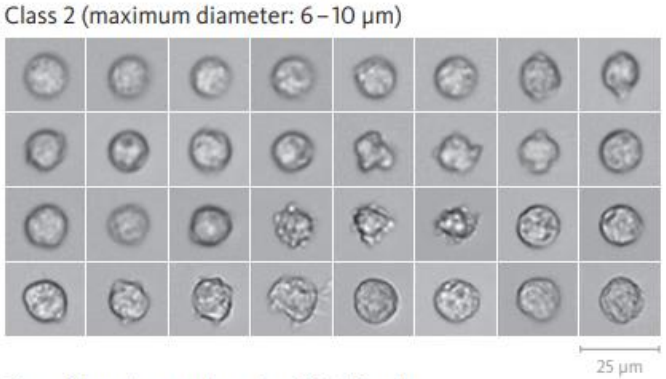
Class 7 (maximum diameter: 101 – 151 µm)

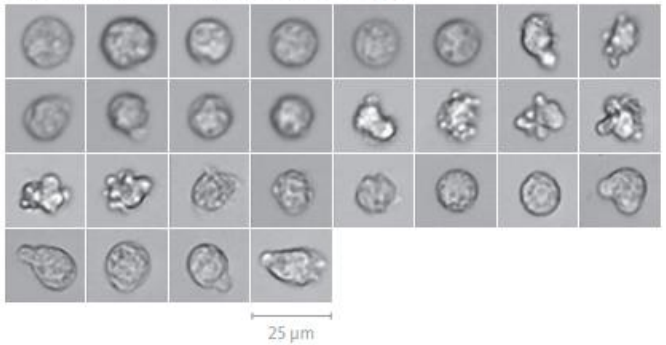


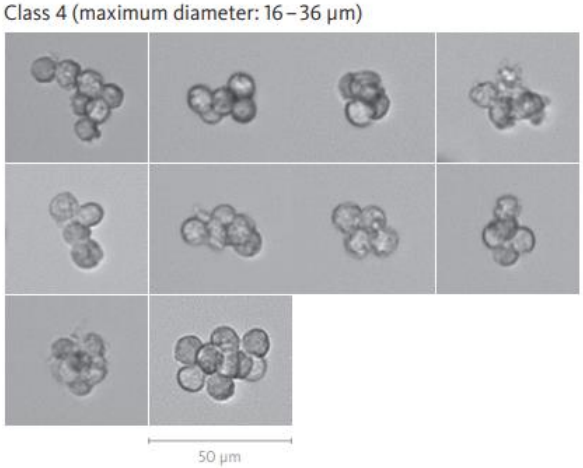
- White blood cells



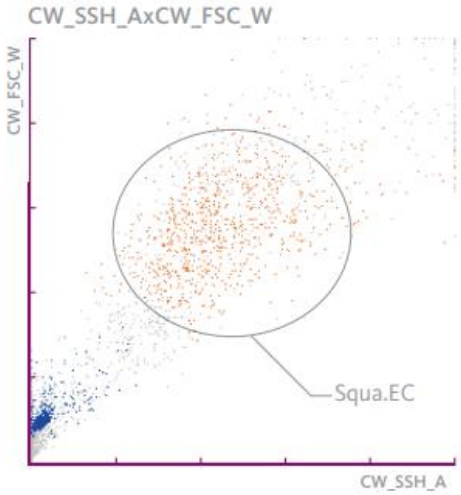




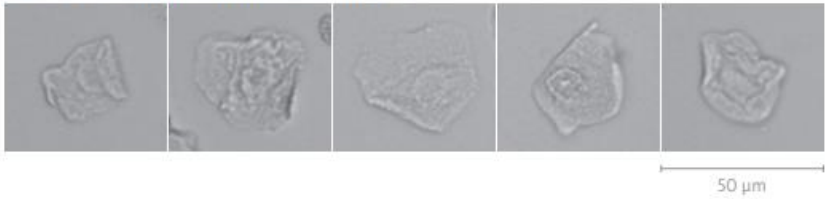




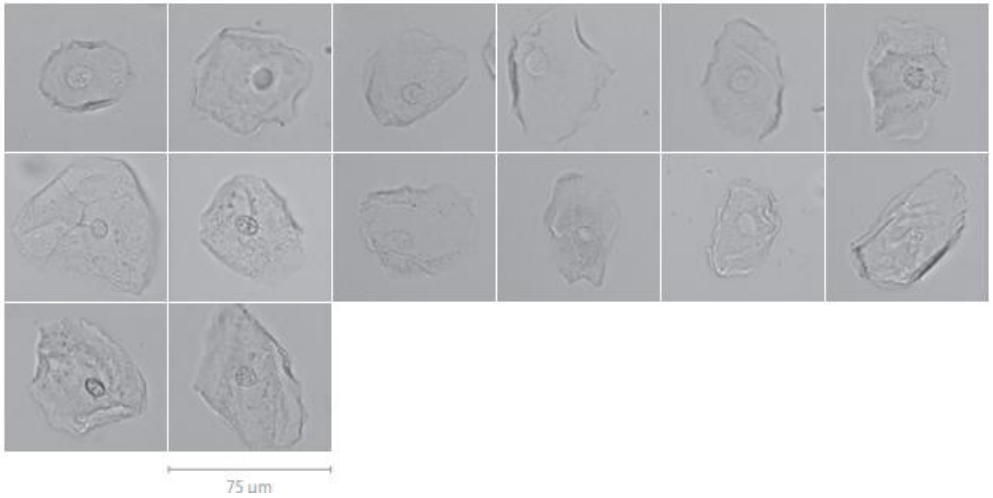
- Squamous epithelial cells



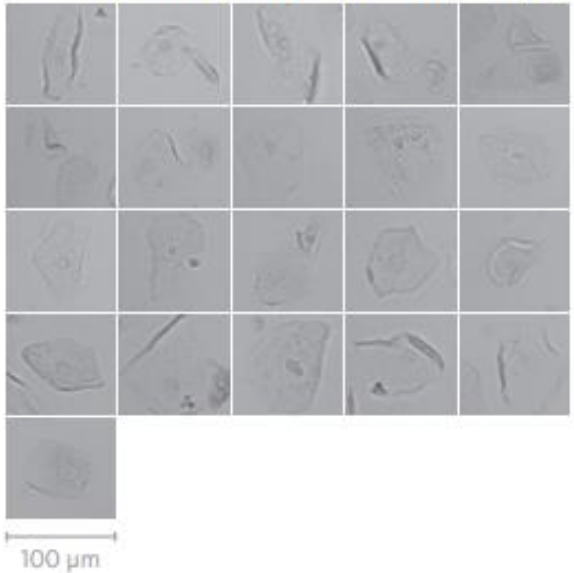
Class 4 (maximum diameter: 16 – 36 µm)



Class 5 (maximum diameter: 36 – 71 µm)

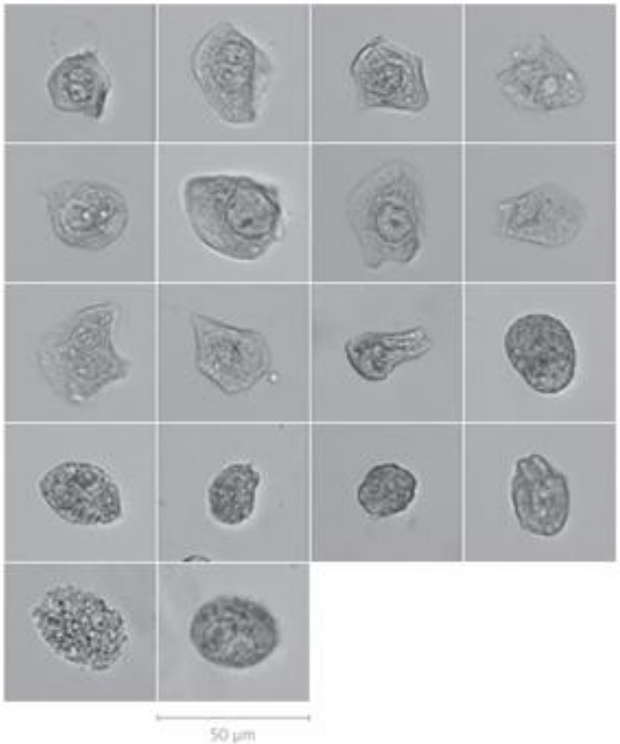


Class 6 (maximum diameter: 71 – 101 µm)

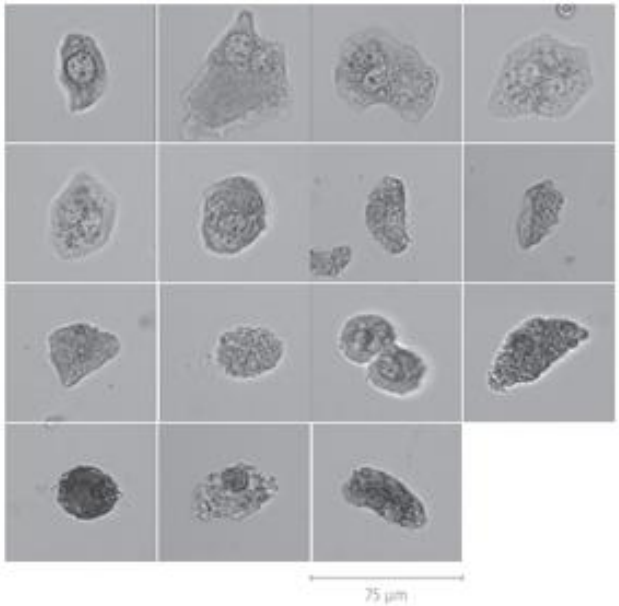


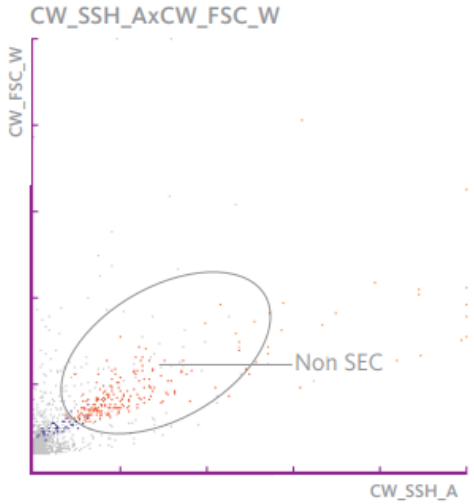
- Non-squamous epithelial cells

Class 4 (maximum diameter: 16 – 36 µm)



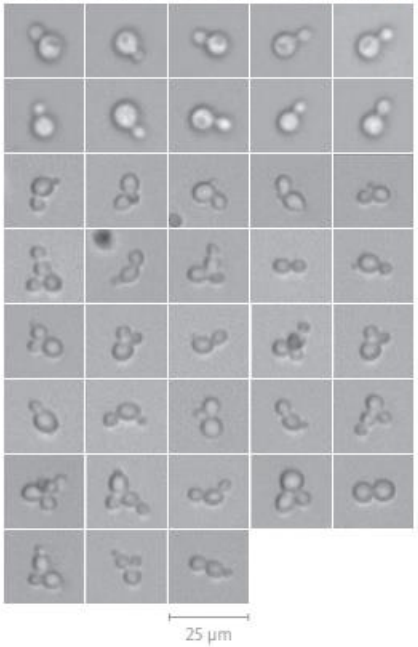
Class 5 (maximum diameter: 36 – 71 µm)



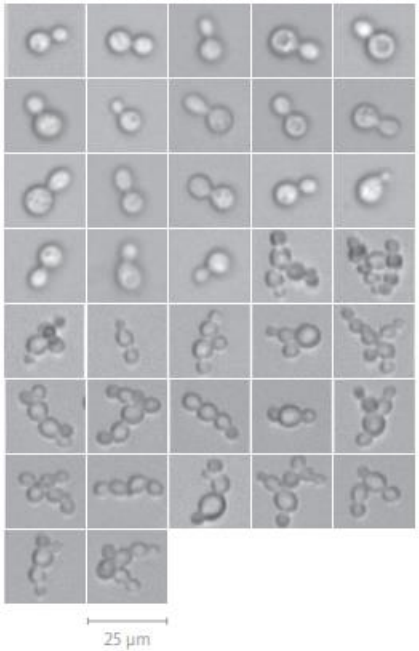


- Yeast-like cells

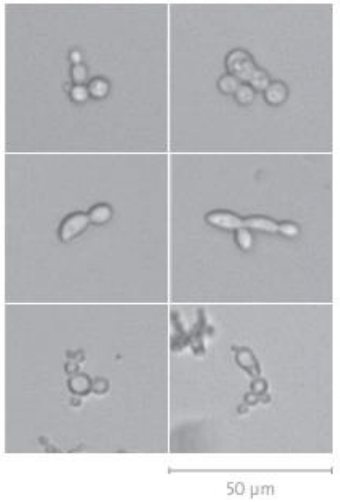
Class 2 (maximum diameter: 6 – 10 µm)

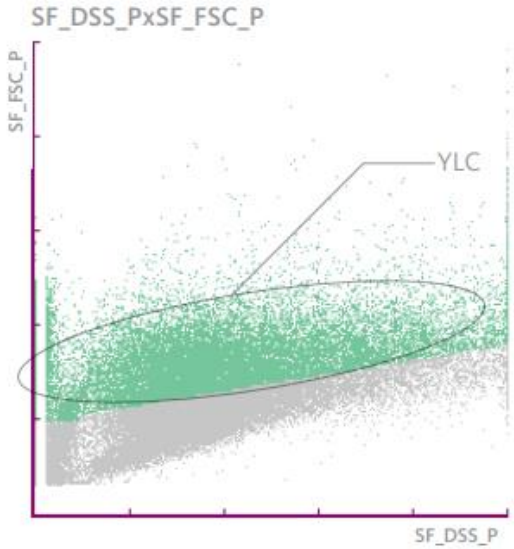


Class 3 (maximum diameter: 10 – 16 µm)

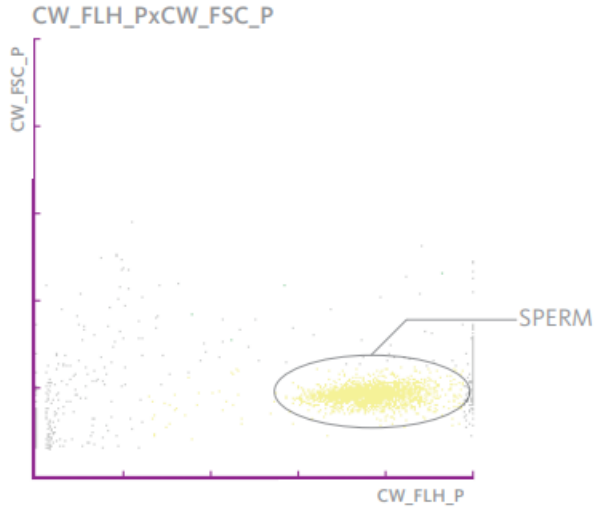


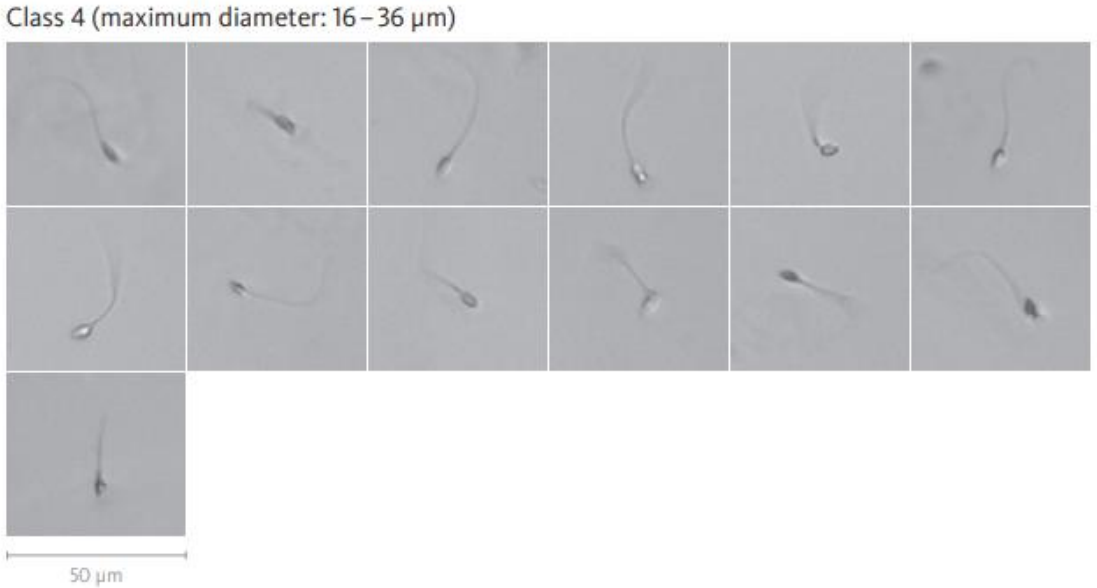
Class 4 (maximum diameter: 16 – 36 µm)



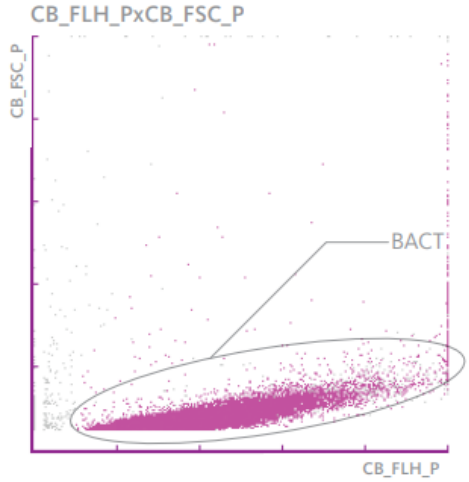


- Spermatozoa

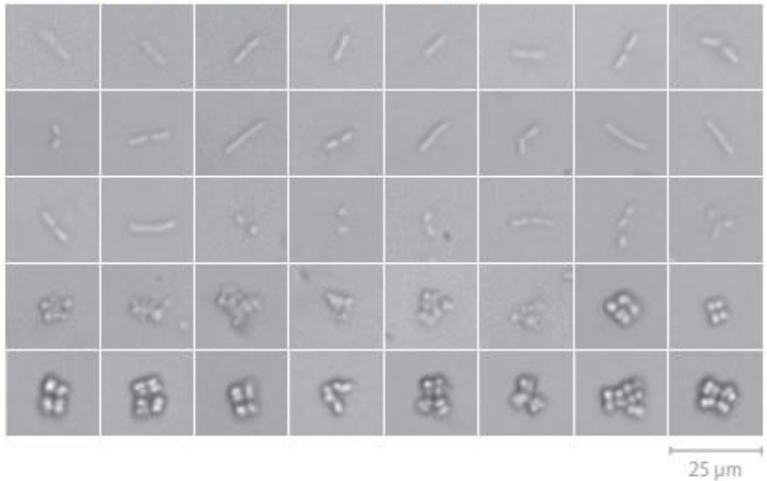




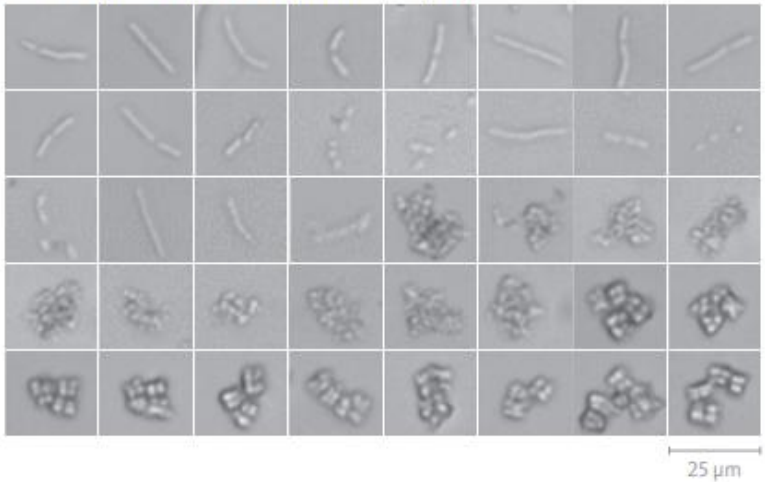
- Bacteria



Class 2 (maximum diameter: 6 – 10 µm)



Class 3 (maximum diameter: 10 – 16 µm)



Critically Appraised Topic

Integration of digital microscopy and flow cytometric analysis of solid elements in urine: The best of both worlds and the gate to total automation

- Drop by drop –

Vanhove, Thibault
06/09/2022