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From photons to publications

Principles of scientific imaging and
developments in image integrity checking

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Introduction

Digital images are an integral part of our society and daily life. A steady flow of images is presented to us every day, when reading daily news, booking tickets online, buying groceries, or when scrolling through social media. A viewer has no need to know, and usually no interest in, the underlying architecture of image files. But scientific images constitute precious raw data, the result of hard labor and lengthy preparation, which is carefully analyzed and stored safely. Understanding how image files are designed not only helps scientific researchers work in a secure and efficient manner it also aids to unravel all the information that is stored in the image file.

The ease at which images are stored, shared, and edited, has led to an increase in image manipulation and fraud. The topic of image integrity is indeed a very broad field, which is relevant whenever an image represents a meaningful fact, for example, in courtrooms and news media. In science, an image is proof of an observation. Therefore, for the scientific researcher, the need to verify and show proof of image authenticity is becoming increasingly important. In this document we discuss recent advances in image security.

First, we outline the basics of digital images, what pixels are, and why file design is important for pixel intensity values. The concept of dynamic range is explained and how it is determined not only by the quality of the physical detector but also by the number of digits of the image file, the bit depth. The same logic also holds true for image resolution. The quality and design by the physical optical components determine the image resolution at capture but the number of pixels in the image can be a limiting factor when saving the image file. Just like the number of digits is important for dynamic range, so are the number of pixels important for image resolution.

In the second section, the most common file formats for molecular imaging applications are described. Depending on how images are captured different file formats are used. Some high-end detectors, such as photomultiplier tubes (PMTs), require certain file formats to preserve all captured image data. These file formats apply mathematical functions to the pixel intensity values in a process called compression. When such files are opened specific software decompresses data to analyze the original image.

Next, we discuss viewing and analyzing image data. During analysis different contrast settings are applied to view different sub-sets of the intensity range. This is often a necessity as the human eye can only differentiate between a small range of the grayscale. Thus, with the contrast settings the user views only the intensity range of interest. However, these contrast settings do not change the raw image data, only what is displayed on the screen. Regardless of the display settings, the measured pixel intensity values are analyzed in the same way. In this section we explain some key terms of this analysis, such as the signal-to-noise ratio. After analysis, images are often exported for presentation and publication. We also describe suitable file formats and resolution settings for image export in this section.

Finally, we address the topic of image security. The need to verify the integrity of original raw data images has led to the development of image security tags embedded in image files, so called checksum fingerprints. Software with specially designed algorithms provide hard proof that images have not been altered after capture. They use the same algorithms as other integrity check software, for example, digital vaccination certificates and document signatures. We conclude with good practices for backing up image data, and some general recommendations regarding handling of image files.

Basic properties of digital images

This section describes the basic properties of digital images. Useful terminology is listed in Table 1.

Table 1. Useful terms and definitions

Term	Description
Pixel	Short for picture element, the smallest element in an image.
Intensity	Each pixel has an associated measured intensity value. Arbitrary units are often used as the values depend on the imaging system and its configuration. If a unit is used the imager is calibrated either at factory or by the user.
Optical density (OD)	Unit to measure absorption of light by a sample.
Dynamic range	Range over which the imaging system gives a response to detected photons. Normally measured as the logarithm of the ratio between highest possible intensity value to the smallest measurable intensity step.
Full-well capacity	Amount of charge an individual pixel can hold before becoming saturated.
Pixel intensity scale	The distribution of measurable pixel intensity levels. Some image types have equidistant integer intensity levels, other file formats exhibit a non-linear distribution of levels.
Grayscale image	Image in which the value of each pixel only carries photon intensity information, not any color information. Sensitive high-end cameras typically capture single-channel grayscale images.
Bit depth	The number of bits used to store the measured intensity values of pixels, for example, 16 bits equals 65536 available intensity levels.
Pixel size	Each pixel corresponds to a square (or sometimes a rectangle) of the imaged object. The length of the side of the area is called pixel size, not to be confused with the actual size of each pixel on the chip.
Resolution	The resolving power of an imaging system which is determined by the ability to separate fine details in the image. It is often measured with resolution test targets.
Image file size	The size of a file is determined by the number of pixels of the image, times the bit depth of each pixel, plus any additional embedded text information.
Contrast range	A set intensity range in a histogram for viewing the image on the display, shown as a histogram.
Gamma value	The contrast display scale can have nonlinear scaling, for example, using a contrast function called gamma. Changing the contrast scale in analysis software does not affect image data.
Data compression	The process of encoding information in an image file. Some detectors measure data in log space, for example PMT tubes. Compression of data is applied before saving the image to preserve the intensity levels. When the file is opened, special software is used to decompress the data.
Signal volume	The integrated signal response from a defined area in the image, for example, for a band the sum of all pixel intensities across the entire band area.
Noise	Undesirable, random signal components found in every electronic system. Noise can be reduced by the quality of electrical components, active cooling, detector calibration, and by simply collecting more photons.
Signal-to-noise ratio (SNR)	The ratio of signal intensity to image noise. It is a key parameter to decide the sensitivity of an imaging system.
Security hash algorithm (SHA)	A hash function maps arbitrary strings of data one-way to fixed length output, called checksum, in a deterministic and public manner. It is not possible to de-construct the input from the output.

Pixels, camera detectors, and viewing images

Pixels

A digital image is essentially a two-dimensional array of data. Each data point in the matrix represents a pixel. The pixel measurement value is the intensity of light detected at that pixel area on the detector. Different detectors have a different number of pixels. The image resolution is first and foremost determined by the optical components of the imaging system, for example, the lens used to collect and direct light to the detector. The number of pixels may limit the resolution; therefore, it is desirable to have many pixels at your disposal. However, too many pixels may lead to large files which are difficult to process for normal computers. With the large scan area of the Amersham™ Typhoon™ scanner (40 × 46 cm) single files with 10 µm pixel size are larger than 1 GB! In such cases it is better to divide a large image into smaller images.

Camera detectors

Modern cameras have detectors in the megapixel range, which is more than sufficient for most applications. Imagine the detector chip as an array of buckets (pixels) that collect rainwater (photons). Each bucket in the array is exposed for the same amount of time to the rain. The buckets fill up with a varying amount of water. The number of measured photons depends on the type of detector.

In a CCD the measurement is initiated by pouring water into an empty bucket in the adjacent row (Fig 1). The buckets in this row transfer their water down to a final bucket where it is measured. Likewise, as electric charge fills up the pixels, it is transferred to the serial register and finally read by converting the charge into a number. In a CMOS sensor the pixels are individually read, each pixel is hardwired to the read-out electronics.

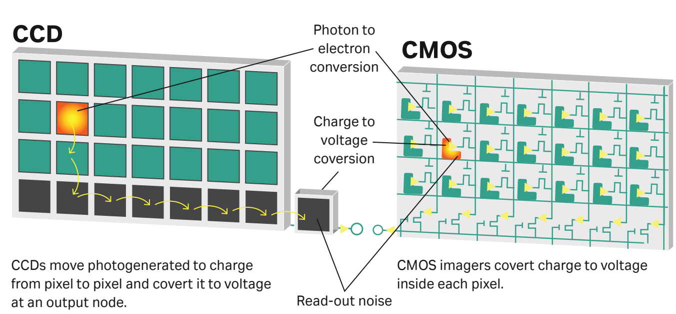

Fig 1. The two most common types of camera detectors, charge-couple device (CCD) and complementary metal-oxide semiconductor (CMOS).

Viewing images

An image is in essence a 3D object, much like a landscape with high pixel intensity mountains and low pixel intensity valleys which we for practical reasons view as a 2D grayscale image during analysis (Fig 2). High end cameras are often single channel, they record the detected light intensity in a single channel. While light sensors for different applications may distinguish among different wavelengths, such as RGB sensors.

Single-channel data is usually analyzed by viewing it in grayscale. However, the human eye can only distinguish a finite number of grayscale levels. Different studies have been performed to decide how many grayscales levels are noticeable and a range of 700 to 900 levels is often given (1). However, many softwares, such as PowerPoint, use only 255 gray levels which is often sufficient for image display (Fig 3). Thus, the image contains many more grayscale levels than we can observe and some range selection for viewing is necessary.

(A)

(B)

(C)

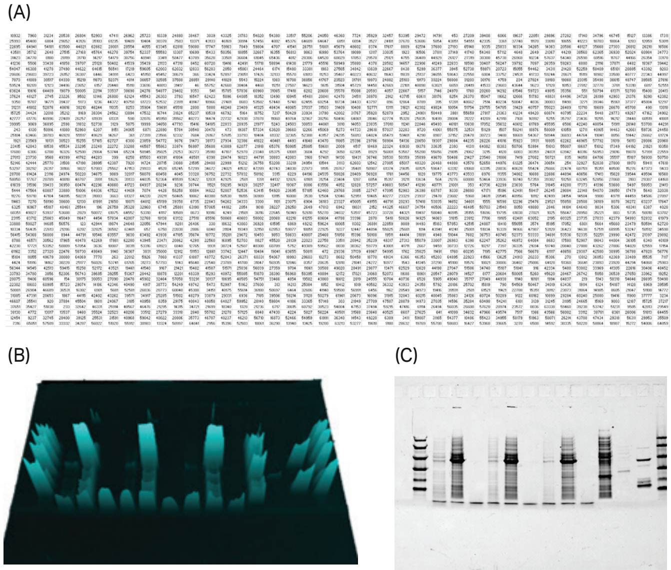

Fig 2. An image is a matrix with intensity values (A) which can be visualized in three (B) or two (C) dimensions. Each matrix element corresponds to a physical pixel on the detector. The intensity values are mapped to a gray or a color-scale to view the three-dimensional data in a 2D image. The screenshots were taken from ImageQuant™ TL analysis software.

If an analysis software is available, we recommend adjusting the contrast range manually, and comparing to the 3D view, to observe all details of interest. If the measured intensity is evenly spread out over the dynamic range a linear contrast scale should be used. However, in some cases it is desirable to focus on the low intensity range, and a non-linear contrast can be applied. To highlight intensity sub-ranges, it is popular to use a multi-color display contrast. Keep in mind that contrast settings never affect raw data, it only changes how the image is displayed on the screen.

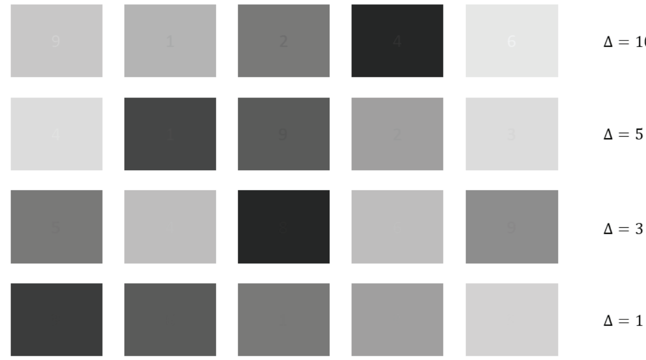

Fig 3. In software applications it is common that the grayscale for viewing has 0 to 255 integer levels, corresponding to an 8-bit file. This is usually sufficient for viewing images as it is difficult to distinguish step differences (Δ) of 1 in such a range. Test if you can see the numbers in this table. Tip: it helps to zoom in. The numbers in the bottom row (3, 6, 1, 3, and 8) are usually not detected.

Dynamic range

Let's have a closer look at the signal intensity values of pixels. When recorded each intensity value may be an integer number or a decimal value. The output from CCD cameras are integer numbers. This simplifies the file handling process greatly as each pixel data point is stored as an integer in standard TIF files. The number of available values is determined by the number of bits of the image file. A 16-bit file allows a maximum of 65 536 values to be stored. Each bit may adopt two values (either 0 or 1), and since there are 16 bits, 2^{16} = 65 536 values. This is termed **bit depth** and may vary for different image files and imaging systems.

The dynamic range is determined by the full-well capacity which is the amount of charge an individual pixel can hold before becoming saturated. This corresponds to the amount of water each bucket can hold. In theory, the pixel dynamic range is defined as the logarithm of the maximum value (65 535) divided by the minimum measurable value and corresponds to 4.8 orders of magnitude. The actual dynamic range is limited by background and device off-sets.

For CCD cameras the measured intensity values are always integer numbers. However, in actual applications another subtle parameter comes into play as analysis measurements are always performed using many pixels. If we take the average of multiple integer pixels values, we get decimal average values. Figure 4 illustrates ensemble averaging and shows that the dynamic range of one single pixel is not the whole story, in actual applications many pixels are always used. Therefore, additional terms to describe pixel variation across the image are needed and the two most important terms, background and noise, which will be explained in detail in the ***Analysis and Export*** section.

10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
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10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10
10	10	10	10	10	10	10	10	10	10	10	10	10	10	10	10

$$\textit{average signal}_{raw} - \textit{average background} = 10.53 - 10 = 0.53$$

Fig 4. An example of ensemble averaging of pixels. In this case, the signal from a 100-pixel large area is of interest (blue area). Some pixels have integer values of 11 and some 10, and the average value is 10.53. After subtraction of the background 10 (white area) the average signal intensity is in fact 0.53, less than the one-step interval of integers. In practice, such weak signals are often obscured by noise and background gradients.

Multi-channel color images

Next, we consider the common case when many images are captured of the same sample object, for example, using different light and filter combinations. Each image is captured as a single channel grayscale image and analyzed individually. But for viewing and making presentations it is useful to make false color images by assigning a color to each image (each channel) and merging them. Only three primary colors (red, green, and blue) are needed to make a full color image (Fig 5). The advantage to this technique is the efficient and striking way it shows differences between images.

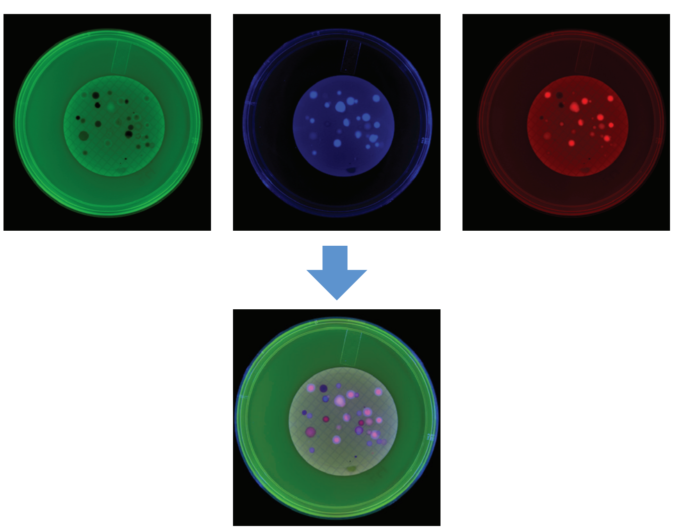

Fig 5. Example of a multi-color image taken using Amersham™ ImageQuant™ 800 Fluor imaging system. Single-channel fluorescence images of colonies on a membrane from environmental sampling in a lab were merged. The different fluorescence properties of these colonies (visualized as black, pink, blue, red) indicate that they come from different bacterial species.

File formats

The diversity of modern cameras and scientific imaging devices has created a plethora of different image file formats. Some are open and well designed, while some are proprietary and non-transparent. This multitude of different file types risks that files are wrongly processed, leading to erroneous interpretation of data. Therefore, the imaging industry should strive to set universal file formats.

The more transparent and open the image file design is, the more the user will benefit from it as software can freely compete in terms of analysis capabilities. However, many closed proprietary file formats are still used. Below we explain some of the most used standard file formats for digital images.

The TIF file

For camera applications one of the most popular file format is the TIF (or TIFF) file. TIF stands for Tag Image File and is based on a standard dating back to 1992 (2). The format was created by the Aldus Corporation for use in desktop publishing and subsequently updated by Adobe Systems after the acquisition of Aldus. TIF was originally created as an attempt to get the scanner industry of the mid-1980s to agree on a common image file format, in place of many proprietary formats. This is just as relevant today.

A TIF file consists of both image data and tags. The tags are numbered and contain meta data in specified data fields. The TIF standard allows developers to use these tags to include information about how the image was created, such as, imager settings, time created, and by whom. This is important information, for example, for an audit trace in a GxP environment. It also allows the inclusion of security tags to ensure that the files are not tampered with or modified in any way. This security feature will be described in more detail in the ***Image Security*** section.

To summarize, the most useful features of the TIF files are:

- Not restricted to specific imaging systems or computer hardware
- Do not favor specific operating systems, file systems, compilers, or processors
- May include one or multiple image files
- Allow for the inclusion of private or special-purpose information, stored in tags. The embedded tags may store information about how the image was captured and how the image should be processed.

In some cases, there is an actual need for special file formats, and this is when special compression schemes are used to fully preserve the measured data. In these cases, you can also benefit from an open and transparent description of how data is stored.

Data compression — GEL and IMG files

Camera detectors, such as CCDs and CMOS, detect photons in a linear fashion. The measured number of photoelectrons is proportional to the number of photons detected. But the output from PMT detectors is often measured in log space, the measured value is proportional to the logarithm of detected photons. This means that the distribution of intensity levels, the intensity scale, has a log distribution. This has an important consequence, the raw data of PMT detectors has many, many more intensity levels at low light levels, which is great when the user wants to measure small changes in light intensity levels from a sample. This is similar to how the eye perceives light. This phenomena of the just noticeable difference for humans being lower at low light intensity levels is known as the Weber's law.

To preserve the intensity scale of raw data from scanner PMTs two unique file formats have been designed. One originated from the scanner manufacturer Fujifilm and is named the IMG file format. The other is from the scanner manufacturer Molecular Dynamics and is named the GEL file format. Both formats rely on compression using mathematical functions when the file is saved. To open the files the same mathematical function must be known to decompress data correctly. The process is illustrated in Figure 6. The many low light decimal levels in these file formats help lower the detection limit and expand the dynamic range. Thanks to the design of these file formats, it is possible to measure minute changes in light intensity with great accuracy.

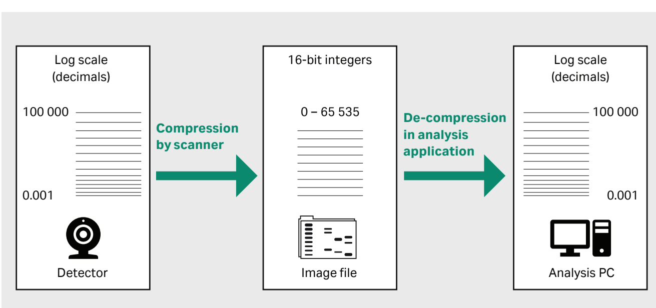

Fig 6. When using PMT detectors it is common that recorded intensity data has a log distribution. This means that there are many more intensity levels at low light levels, with many decimals, which is useful to measure small changes in signal intensity using compatible analysis software.

Common file formats used by Cytiva imagers are listed in Table 2. Note that some are designed for analysis and some for making presentations and figures for publication. Also note that the analysis software is only compatible with some file types. For example, the GEL and IMG file types are read using the ImageQuant™ TL software.

Table 2. Common image file formats

File format	Intended use	Compatibility
TIF	Analysis	All analysis software that can read TIF files
TIF with OD units	Analysis	ImageQuant™ TL
GEL	Analysis	ImageQuant™ TL to decompress square root encoded data
IMG	Analysis	ImageQuant™ TL to decompress log encoded data
JPEG	Presentations and figures for publication	Any image viewing software
PNG	Presentations and figures for publication	Any image viewing software
BMP	Presentations and figures for publication	Any image viewing software

Analysis and export

Now that we know the importance of pixels and how data is stored in an image file, we discuss the analysis of images and how to describe the properties of pixels. It is good practice to first inspect the image for example, using the ***Toolbox*** module of ImageQuant™ TL analysis software.

One way to inspect images is using line profiles for a quick determination of signal and background levels. If a more quantitative analysis is to be performed it is possible to analyze areas in detail, for example, using rectangular areas and measuring the average intensity after background subtraction (Fig 7). The average signal can be compared to the background noise, which is the standard deviation of the background.

Fig 7. Area analysis in ImageQuant™ TL includes analysis of region of interest and background. The area is measured in units of pixels (width times height). After background subtraction, the mean pixel intensity is compared to the background and the background noise level.

Useful image analysis terms

The following terms are commonly used for the analysis of images are defined in Table 3.

Table 3. Common terms used for the analysis of images

Term	Definition
Signal volume	The integral of pixel intensity for all pixels in the region of interest
Area	The area is often measured in units of pixels (width times height).
Background	Signal in area without any sample
Noise	Variation in background signal, measured as the standard deviation

The term signal volume may sound out of place but considering that an image is a 3D object, it reflects the fact that the total signal is the integral of all pixel intensity values in the area of interest. In image analysis there are two common terms which describe the quality of an image: signal-to-background ratio and signal-to-noise ratio (SNR). SNR is often used when detecting weak signals near the sensitivity limit, to ensure that the signal is not noise or a background artefact.

Export options

Once the analysis has been completed, it is often desirable to export a snapshot of the image with the chosen contrast settings, and with the created analysis objects. When exporting an image, you may select a suitable file type and resolution. The resolution setting is important so as not to miss any details from the original image. Also, it allows the user to meet any publishing requirements. But these files should not be used for further analysis since the exported image may lack information compared to the original raw data.

Image security

Security hash algorithms

The last few decades have seen tremendous developments in information security. Methods and algorithms for safeguarding digital assets have improved and evolved into standards. Secure Hash Algorithms (SHA) are a family of such standard cryptographic hash functions which are published by the National Institute of Standards and Technology (NIST). SHAs are commonly used to ensure that digital information has not been altered, deliberately or accidentally. The popularity and importance of these algorithms stem from their usefulness in making unique fingerprints of data, and their use has grown exponentially.

A hash function computes a fixed-length message digest one-way from arbitrary length data in a deterministic and public way. The algorithms use public mathematical functions, and the process can be repeated, with the same image always yielding the same results. A unique property of these calculations is that they are one-way. It is not possible to do the calculations backward or reverse the calculations to construct the input from an output (Fig 8).

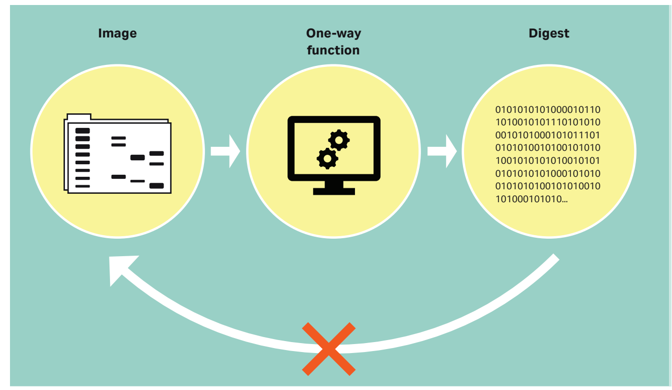

Fig 8. One-way secure hash algorithms compute a fixed-length message digest from arbitrary length data. An input of varying length is used to create the output, however, the output cannot be used to de-construct the input.

The one-way information flow of hash algorithms is remarkable, and it has revolutionized many areas of data security by providing an efficient way to create data fingerprints. For example, the commonly used SHA-256 algorithm, provides a 256-bit output in the form of 64 hex blocks of bits. It is deterministic, meaning that the same input will always generate the same output. The algorithm is open so that anyone can generate this output. There is also a “butterfly effect” built into the algorithms, which means that a small change in the input has an avalanche effect and produces a completely different output. Thus, the output is randomized in the sense that it is not possible to predict the outcome based on the input or a small change of the input. In fact, the 256-bit output number space is so huge that each created fingerprint number has never been observed before, each fingerprint is unique.

The use of SHA is now commonplace in many applications to ensure authentication of a wide variety of digital objects, for example proof of vaccination, digital signatures, and digital currency. The topic of image authenticity is especially important, for example, for images that are used in the news or in courtrooms. In science, images constitute raw data and there is a trend that scientific journals request raw data to be submitted with articles.

To meet these demands, imaging systems have been designed to start producing images with digital fingerprints. We first introduced images with digital fingerprints in ImageQuant™ 800 GxP imaging systems. Our recent development of the stand-alone software, Cytiva Image Integrity Checker, which can read digital fingerprints generated by Typhoon™ laser scanner platform, will help scientists and article reviewers to confirm that raw data is correct. The workflow for image analysis is shown in Figure 9.

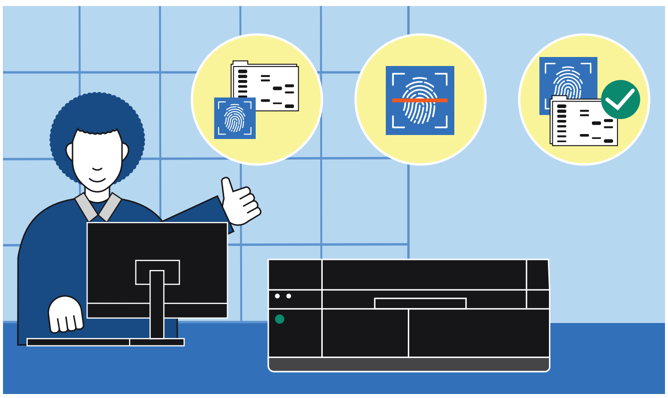

Fig 9. Captured images are saved with a digital fingerprint. The fingerprint is generated by the control software using a secret key. The same key is also used by the stand-alone software to read the fingerprint and verify that the image has not been altered. Furthermore, the stand-alone software application is authenticated at installation.

Storage of data

Making backup copies of image files is good practice. In general, it is always possible to ***Copy*** and ***Paste*** image files. When image files are copied identical files, with identical image properties, are created and this does not affect the authenticity of data. It is also worth mentioning that modifying the file name will not affect the fingerprint of the image file. The fingerprint and the file name are not connected, which is good to know when making backup copies of image data.

The ease at which files can be copied and stored is indeed a very useful property of digital images. In contrast to physical images, digital images can, in principle, be stored forever. If the current SHA algorithms withstand the test of time, we can in the future go back and check if image files are the original images. Perhaps we will look back and describe digital images as captured before or after the advent of digital fingerprint algorithms. The transition to include digital fingerprints to images at capture is ongoing. And the makers of imagers will strive to set open and transparent file format standards to help users verify images, now and in the future.

References

1. Kimpe, T., Tuytschaever, T., Increasing the Number of Gray Shades in Medical Display Systems—How Much is Enough? *J Digit Imaging.*2007;20(4): 422-423. doi: 10.1007/s10278-006-1052-3.
2. *TIFF.* Edition 6.0. Mountain View, CA: Adobe Developers Association; 1992.

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