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Oxidation Monitoring by Fluorescence Spectroscopy Reveals the Age of Fingermarks**

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Abstract: *No forensic method exists that can reliably estimate the age of fingermarks found at a crime scene. Information on time passed since fingermark deposition is desired as it can be used to distinguish between crime related and unrelated fingermarks and to support or refute statements made by the fingermark donors. We introduce a non-contact method that can estimate the age of fingermarks. Fingermarks were approached as protein–lipid mixtures and an age-estimation model was build based on the expected protein and lipid oxidation reactions. Two measures of oxidation are required from the fingermark to estimate its age: 1) the relative amount of fluorescent oxidation products 2) the rate at which these products are formed. Fluorescence spectroscopy was used to obtain these measures. We tested the method on 44 fingermarks and were able to estimate the age of 55% of the male fingermarks, up to three weeks old with an uncertainty of 1.9 days.*

Accurate recording and analysis of fingermarks is essential to the processing of a crime scene. Establishing the time passed since fingermark deposition can be of crucial importance. Crime-scene investigators can use it to find and select relevant evidence and to discard marks that are not crime related. Also, such information can be used to assess statements from witnesses, victims, and suspects.[1] Currently age estimation of fingermarks is impossible. In the past, age has been estimated from of the quality of the print and the ease of development with for instance, dactyloscopic powder, but fingermarks appearing "fresh" were in fact old.[2] The main progress made so far is classifying fingermarks as younger, or older than 5 h using white-light imaging.[3] The obstacle that has prevented the development of an age-estimation method for fingermarks is their highly variable chemical composition which affects the aging process.[4] In contrast, the age estimation of blood stains is possible based on the quantification of the oxidation products of hemoglobin.[5] We propose that for fingermarks oxidation processes can also be used to estimate their age.

Generally, fingermarks contain proteins and lipids.[4] Tryptophan-containing proteins (Tryp) have been proposed to be the main contributor to fresh fingermarks' autofluorescence (Figure 1 A).[6] When exposed to air, unsaturated lipids oxidize and form reactive oxidation products (LipOx),

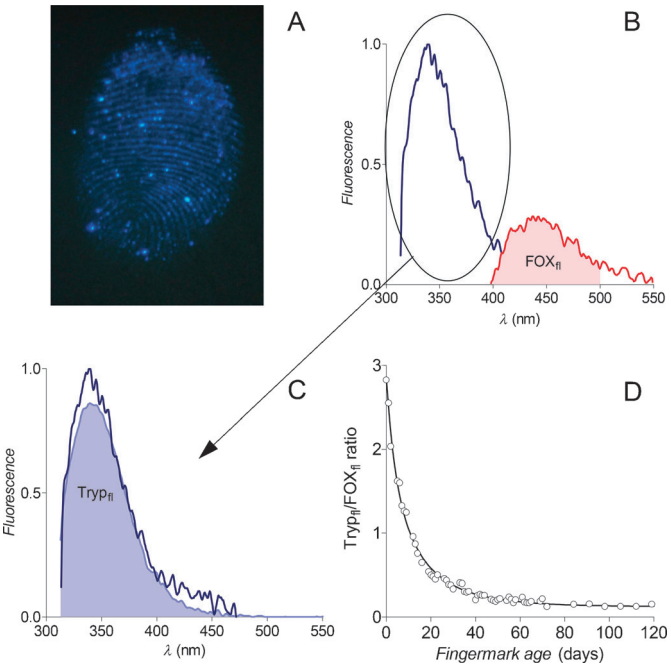

Figure 1. Fingermark age-estimation procedure. A) Autofluorescence image of a fingermark illuminated with 365 nm light. B) Tryp and FOX fluorescence emission spectra. Blue line: excitation at 283 nm, red line: excitation at 365 nm. Shaded area: integrated area for FOX$_{fl}$ determination. C) Fit of reference fluorescence emission spectrum (lavender line) to the measured Tryp fluorescence emission spectrum (blue line) as shown in (B). D) ○: Tryp$_{fl}$/FOX$_{fl}$ ratio of an aging fingermark, black line: fitted aging curve.

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which react with proteins to form fluorescent oxidations products (FOX) [Eq. (1)]:[7]

$$\mathrm{LipOx} + \mathrm{Tryp} \rightarrow \mathrm{FOX} \tag{1}$$

The extent of oxidation in protein–fat emulsions follows from the decrease of fluorescence intensity of Tryp and the increase of FOX fluorescence.[7] Depending on the fingermark composition, not all Tryp may be converted by LipOx. We therefore split Tryp into a part which will eventually react ($\mathrm{Tryp_R}$) and a part which will not be converted ($\mathrm{Tryp_C}$). As LipOx is generated by oxidation of unsaturated lipids and consumed by oxidation of proteins, we assume the concentration of LipOx to remain constant, indicated by $[\mathrm{LipOx}]_0$. Thus, the reaction follows pseudo first-order reaction dynamics [Eq. (2)]:

$$-\frac{\mathrm{d}[\mathrm{Tryp_R}]_t}{\mathrm{d}t} = k'[\mathrm{LipOx}]_0[\mathrm{Tryp_R}]_t = k[\mathrm{Tryp_R}]_t \tag{2}$$

Equation (3) thus follows:

$$\begin{aligned}[\mathrm{Tryp}]_t &= [\mathrm{Tryp_R}]_0 e^{-kt} + [\mathrm{Tryp_C}] \text{ and} \\ [\mathrm{FOX}]_t &= [\mathrm{Tryp_R}]_0\left(1 - e^{-kt}\right) + [\mathrm{FOX}]_0\end{aligned} \tag{3}$$

FOX is generated as described by Equation (1) and $[\mathrm{FOX}]_0$ is the concentration of FOX already present in the fingermark at the time of deposition ($t = 0$). From Equation (3) the aging function is derived [Eq. (4)] (Supporting Information, Section 2):

$$f(t) = \frac{[\mathrm{Tryp}]_t}{[\mathrm{FOX}]_t} = \frac{(f_0 - f_\infty)e^{-kt} + f_\infty(f_0 + 1)}{-(f_0 - f_\infty)e^{-kt} + f_0 + 1} \text{ (aging function)} \tag{4}$$

with f_0 being the value of $f(t)$ at $t = 0$ and f_∞ is given by Equation (5).

$$f_\infty = \lim_{t\to\infty} f(t) = \frac{[\mathrm{Tryp_C}]}{([\mathrm{Tryp}]_0 - [\mathrm{Tryp_C}]) + [\mathrm{FOX}]_0} \tag{5}$$

As [Tryp] and [FOX] cannot be determined in fingermarks, we assumed them to be proportional to their autofluorescence intensity integrated over the wavelength range 313–550 nm ($\mathrm{Tryp_{fl}}$; excitation at 283 nm) and 400–500 nm ($\mathrm{FOX_{fl}}$; excitation at 365 nm).[7] Excitation–emission contour plots obtained from fresh and aged fingermarks confirmed the accurateness of the chosen wavelengths (Supporting Information, Section 4, Figure S2).[8]

Fluorescence originating from non-protein fluorophores may lead to a Tryp overestimation. We therefore constructed a reference spectrum using six fingermarks washed with a chloroform/methanol mixture known to elute fluorescent oxidation products and other unknown fluorescent components while retaining proteins.[9] This reference spectrum was fitted to the Tryp fluorescence spectra using a weighted least-squares fitting method. The area under the curve of the fitted spectrum was $\mathrm{Tryp_{fl}}$ (Figure 1 C). The $\mathrm{Tryp_{fl}}/\mathrm{FOX_{fl}}$ ratio could now be calculated and plotted against time (Figure 1 D).

For age estimation, the $\mathrm{Tryp_{fl}}/\mathrm{FOX_{fl}}$ ratio was measured for the first time at (an unknown) t_0 days after fingermark deposition and subsequently for several days, thereby generating multiple values for $f(t_0+t_{\mathrm{measured}})$, where t_{measured} is the time of subsequent measurements. The parameter f_0, the $\mathrm{Tryp_{fl}}/\mathrm{FOX_{fl}}$ ratio of fresh fingermarks, was established from 23 fresh fingermarks (3.85 ± 3.48, Supporting Information, Figure S1). By fitting the aging function [Eq. (4)] to the data points using a nonlinear least-squares method t_0, k, and f_∞ could be estimated. The first measurement of an unknown fingermark ($t = t_0$) was used to assess the suitability of that fingermark for age estimation. We included three criteria (Supporting Information, Section 3): a,b) $\mathrm{Tryp_{fl}}$ and $\mathrm{FOX_{fl}}$ had to exceed a predefined minimum; c) fitting the reference spectrum to the measured Tryp fluorescence spectrum had to yield a positive R^2 value. The Tryp inclusion criteria (a,c) were incorporated to ensure dominant contribution of protein fluorescence over background fluorescence. They were only applied to the first measurement at $t = t_0$ as Tryp is expected to disappear and FOX to appear in subsequent measurements. Aging curves were included for age estimation when a) $R^2 \geq 0.85$, b) a minimum of six non-constant data points were present, and c) the ratio of the first measurement ($t = t_0$) was at least 0.2. A signal-to-noise limit was set at 3.92 (Supporting Information, Section 3). Stacked fingermarks were used to obtain a stronger fluorescence signal; volunteers deposited five or ten fingermarks (one for each finger) on top of each other.

A set of 22 male and 22 female fingermarks was used to test our method. First, the complete time series (ranging from 65 to 176 days in length) of each fingermark was used for fitting. Then, the first time point was excluded from the time series and the aging function was fitted again and so forth. In this way, the data from each fingermark were reused to simulate increasing ages for t_0, varying from 1 hour to 5 months. Twelve fingermarks were suitable for age estimation, however more than twelve data points were generated based on the simulation of increased ages (Figure 2).

All fingermarks suitable for age estimation were derived from male donors due to the low fluorescence signal retrieved from female fingermarks: 77% of the fresh female fingermarks did not display sufficient $\mathrm{Tryp_{fl}}$ fluorescence as opposed to 27% of the male fingermarks. Age assessments were possible up to three weeks after deposition with a median uncertainty of 1.9 days. To demonstrate that the

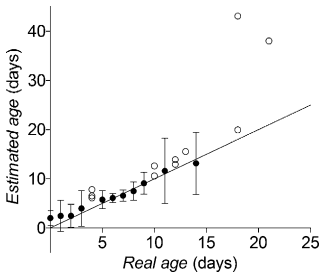

Figure 2. Age-estimation results for twelve fingermarks. By reusing the data from a single fingermark, the method was tested for a wide range of ages. ●: median average of at least four data points, ○: single results. Error bars: inter-quartile range. The straight line was added as a visual guide.

aging rate depends on the unsaturated lipid content, six volunteers deposited both natural and squalene-spiked fingermarks (fingertips rubbed with squalene prior to deposition). Squalene is a highly unsaturated lipid present in fingermarks known to readily oxidize.[10] Fingermarks typically contain cholesterol and unsaturated fatty acids, such as oleic acid, which are also expected to oxidize and thus to contribute to the aging process.[10] Squalene clearly accelerated the fingermark aging rate (Figure 3, Supporting Information, Section 5 and Figure S3).

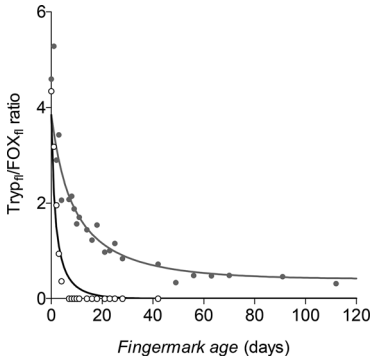

Figure 3. Effect of squalene on fingermark aging rate. Typical example of aging natural (○) and squalene spiked (●) fingermark. Lines represent fitted aging curves.

Fifty-five percent of fingermarks from male donors were suitable for age estimation versus none from female donors. We attribute this to the lower excretion of skin components by women.[11] This does not imply inappropriateness of our ageing model, but shows more efficient techniques for deposit collection are required. We expect that fingermarks displaying little to no fluorescence in our study are non-detectible in forensic practice as well. Given the gender-bias in crime statistics leaning towards males, the apparent inability to date female fingermarks does not compromise the applicability of our method.[12] Traces such as semen and vaginal fluid can also be approached as protein–lipid emulsions. Preliminary experiments with semen yielded similar aging curves as described in this work.

The use of fluorescence to establish the time passed since deposition was explored over thirty years ago when a "red shift" (which befits the formation of FOX) towards longer fluorescing wavelengths was observed for aging fingermarks.[13] The inter- and intra-person variability was too large for quantitative age inference. We tackled the variability issue by measuring the aging rate. Corrections for environmental conditions are needed to complete our age-estimation model. Preliminary experiments demonstrated that although the aging rate increases at higher temperature, our method can still date fingermarks (Supporting Information, Section 6, Figure S4). The deposition substrate also affects the aging rate: for example a faster degradation of squalene and cholesterol in fingermarks on glass compared to those deposited on microfilter paper has been reported.[10c] Our method includes the aging rate in the age estimation and thus accounts for substrate effects. Strong substrate fluorescence and the requirement to monitor the aging process over time are limitations to our method. Translation of the age-estimation model to the crime scene can follow two routes. 1) Optimization of the experimental set-up to minimize noise will reduce the required monitoring time and enable onsite measurements. 2) The requirement of monitoring the oxidation process over time can be abandoned if FOX components generated at different rates are identified. Establishing ratios of these components relative to un-oxidized proteins for example, by mass spectrometry imaging or specific labeling techniques,[14] will enable age estimation in one single measurement.

In conclusion, we have successfully introduced a fingermark age-estimation method. Fluorescence spectroscopy was used to measure the degradation and generation rate of proteins and oxidation products, respectively, in aging fingermarks. We were able to estimate the age of 55% of the fingermarks from male donors up to three weeks old with an uncertainty of 1.9 days. The method presented is not limited to fingermarks but serves as a template for the age estimation of all protein and lipid containing traces such as semen, vaginal fluid, or even tears.

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